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Proton Activation in Space Vehicles

Paul W. Todd

In view of present investigations with space vehicles, it is important to determine the consequences of induced radioactivity in the inner radiation belt of the earth. It is of particular interest to know whether induced radioactivity has a significant effect on radiation detectors, biological specimens, and ultimately on man during space vehicle flight through the inner Van Allen belt.

THE NATURE OF THE RADIATION

The Van Allen radiations are distributed in two large equatorial belts about the earth, the inner of which has its maximum intensity at about 3,600 km from the surface of the earth and 10,000 km from the center of the earth (1). Both belts are characterized by very intense electron radiation and less intense proton fluxes (1-3).

For the present purpose only the proton flux in the lower belt is considered, as it has been reasonably well characterized (4-7). Furthermore, the proton intensity in the outer zone is negligible in comparison with that in the inner zone.

From data obtained by space vehicles (1,8) it is possible to construct a rough distribution of proton fluxes as a function of distance from the earth. Utilizing total counting data and estimated proton fluxes of 1,100 protons/cm^2-sec at 1,200 km (5), 20,000 protons/cm^2-sec (of energy greater than 40 MeV) at 3,600 km (1), and corrections for unobserved protons due to energy distribution (6), the flux distribution of protons with energy greater than 10 MeV can be estimated.

After corrections are made for unobserved protons, and the known fluxes at 1,200 km and 3,600 km are used as normalization points, data from Pioneer III and Pioneer IV (1) can be used for proton-flux mapping along a path normal to the earth's surface at the geomagnetic equator. This plot is given in Fig. 1.

Since analytic functions are not available for flux distributions and activation cross-sections, it is necessary to obtain a mean effective proton flux by numerical integration of the graph of Fig. 1. When this is done, the

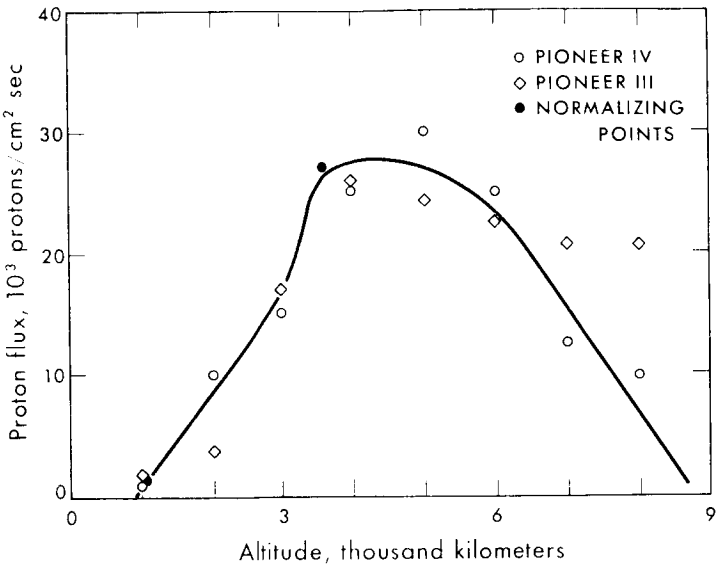

Figure 1. Radial flux distribution of Van Allen belt protons in the equatorial plane from normalized data obtained by Pioneer III and Pioneer IV.

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mean effective proton flux from 1,000 km to 10,000 km is 1.7×10^4 protons/cm^2-sec. It is assumed to be zero outside this region.

For the present purpose, the energy spectrum of the proton flux in these altitudes is assumed to be everywhere that described by Freden and White (6). Because their observations were made at 1,200 km, where the intensity is very low, their spectrum must be corrected to be in accordance with the mean total proton intensity in the entire inner belt. Since the integrated flux at 1,200 km is 0.11×10^4 protons/cm^2-sec, the spectrum of Freden and White is multiplied by 15 and presented in histographic form for convenience of calculations to be presented later. See Fig. 2.

NUCLEAR REACTIONS OF PRIMARY INTEREST

Because the study of activation of all elements by protons in the inner radiation belt would be an undertaking of unjustifiable magnitude, this study is limited to an investigation of the activation of the more important elements carbon, nitrogen, oxygen and aluminum.

As proton excitation functions are not generally available, a survey of the literature was made. It was found that the following radionuclide-producing reactions could be eliminated: $C^{12}(p,\gamma)N^{13}$; $C^{12}(p,+)C^{10}$; $C^{12}(p)Li^8$; $C^{12}(p)He^6$; $C^{12}(p)Be^8$; $N^{14}(p,\gamma)O^{15}$; $N^{14}(p,t)N^{12}$; $N^{14}(p,n)O^{14}$; $O^{16}(p,\gamma)F^{17}$. Most of these reactions occur at negligible rates in comparison to those of the reactions to be discussed (9-25). Sufficient data are not available to justify study of the reactions $Al^{27}(p)N^{13}$; $Al^{27}(p)O^{15}$; or $Al^{27}(p)Be^6$ (16,26,27).

From the general literature, excitation functions were compiled for the

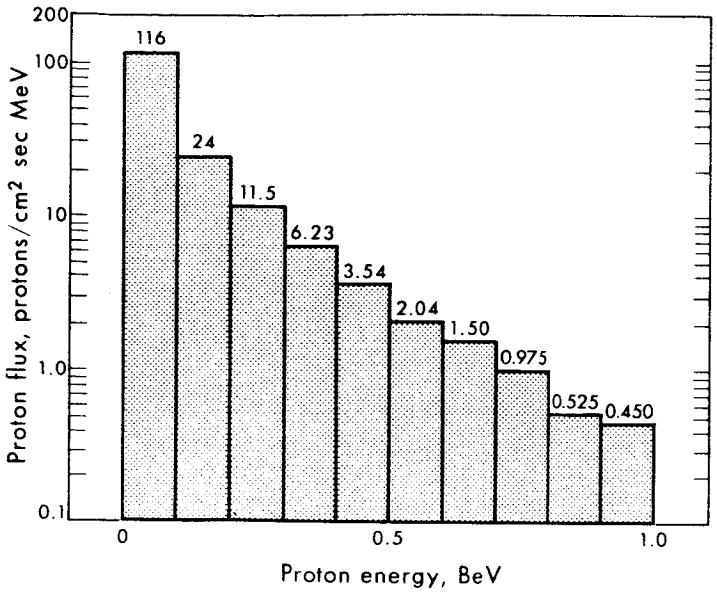

Figure 2. Mean proton flux spectrum in the inner Van Allen belt.

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reactions of interest. Sketches of each are given in Fig. 3 plotted on linear scales for ease in numerical integrations necessary for the determination of specific activity. Dashed portions of the curves indicate regions where no data are available, and known points have been connected. The literature cited in each case in annotated with the graph.

SPECIFIC ACTIVITIES OF THE PRODUCT RADIONUCLIDES

From the effective flux-cross-section product, defined by

$$\overline{\phi\sigma} = \int_0^{1\,\mathrm{BeV}} \sigma(E)\,\frac{d\phi(E)}{dE}\,dE$$

and obtained by numerical integration over 0.1-BeV energy intervals from zero to one BeV, the radioactivity induced in each reaction can be obtained. From the equation

$$\left(\frac{dN}{dt}\right)_0 = \frac{\overline{\phi\sigma}\,N_A}{A}\left(1-e^{-\lambda t}\right)$$

the initial specific activity $(dN/dt)_o$ is obtained directly, where N_A is Avagadro's number, A is the atomic mass of the parent nuclide, λ is the decay constant of the daughter nuclide, and t is the activation time--the 25 minutes required to pass through the inner Van Allen belt (3). The manipulations involved in equations (1) and (2) are given in Table 1 for each of the eleven nuclear reactions under study. The practical applications that follow are based on Table 1.

ACTIVATION OF A PLASTIC SCINTILLATOR

A plastic scintillator used in the artificial earth satellite 1958 ϵ was designed to count electrons of energy greater than 650 keV, protons of energy greater than 10 MeV, and protons of energy greater than 400 keV (2). Thus it is assumed that any radioactivity induced was counted with 100% efficiency.

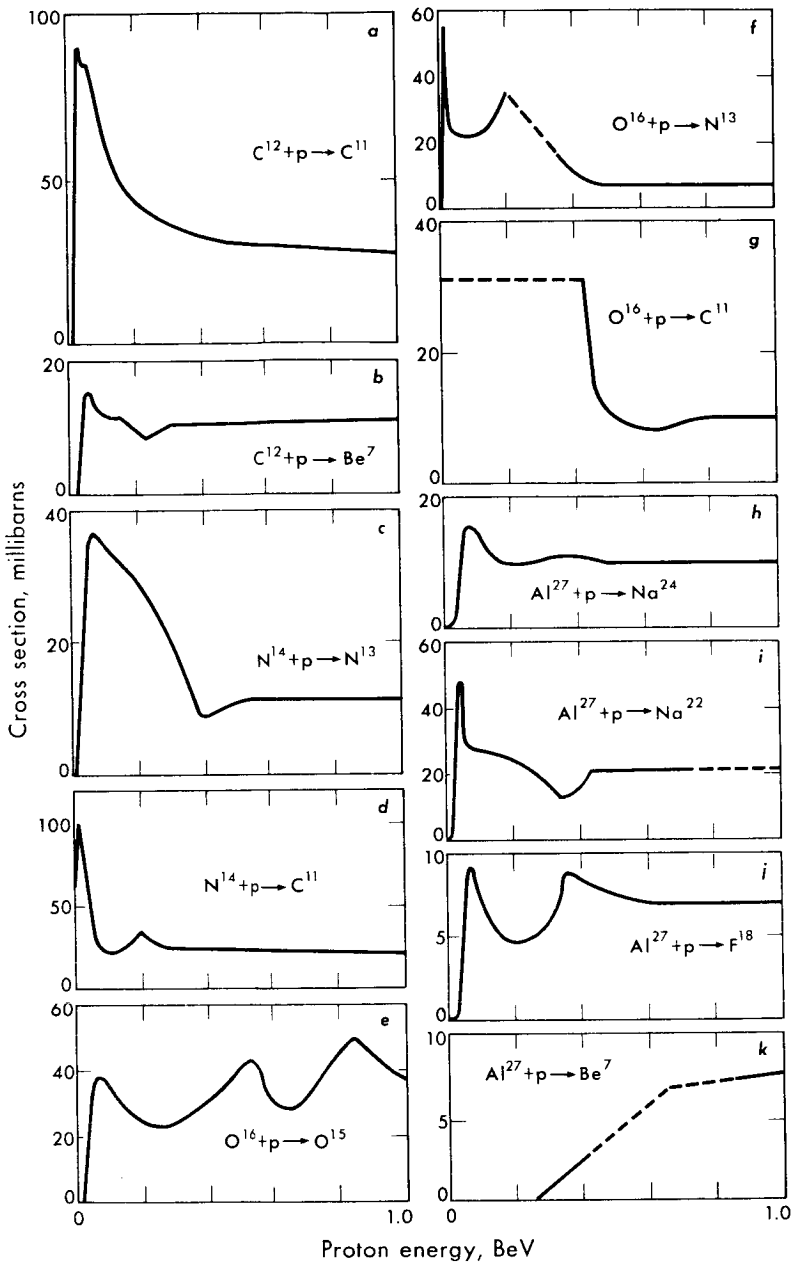

Figure 3. Excitation functions for proton reactions on the nuclei of carbon, nitrogen, oxygen and aluminum. The ordinate is the cross-section in millibarns and the abscissa is the proton energy in BeV. Both are linear scales for ease of numerical integration. The references for the figure are as follows: a. (14,28-40); b. (40-43); c. (14,44-47); d. (14,48-50); e. (14,46,51,52); f. (14,49,51,53,54); g. (14, 54; h. (31,34,36,39,46,54-56); i. (30,39,54); j. (39, 42,57-59); k. (42,43,54).
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The scintillator was 0.762 cm in diameter and 0.178 cm thick. If it had composition similar to that of lucite (3.6×10^{22} atoms of carbon/cm^3), it contained 2.9×10^{21} carbon atoms. Therefore, according to Table 1, it had an initial self-counting rate of 1.56 counts/sec immediately after having traversed the inner radiation belt. This is very negligible in comparison with that recorded at 10,000 km during space flight (3).

Table 1. Specific activities of the daughter radionuclides

Parent nuclide	Daughter nuclide	λ (min^{-1})	$\overline{\phi\sigma}$ (protons/atom-sec)	$(dN/dt)_0$ (dps/g)
C^{12}	C^{11}	.0338	9.43×10^{-22}	27.0
C^{12}	Be^{7}	8.9×10^{-6}	1.42×10^{-22}	negligible
N^{14}	N^{13}	.0693	4.30×10^{-22}	15.0
N^{14}	C^{11}	.0338	7.00×10^{-22}	17.0
O^{16}	O^{15}	.329	4.42×10^{-22}	16.7
O^{16}	N^{13}	.0693	4.02×10^{-22}	12.5
O^{16}	C^{11}	.0338	5.17×10^{-22}	4.12
Al^{27}	Na^{24}	.00077	1.46×10^{-22}	0.021
Al^{27}	Na^{22}	5×10^{-9}	3.55×10^{-22}	negligible
Al^{27}	F^{18}	.00621	7.92×10^{-23}	0.25
Al^{27}	Be^{7}	8.9×10^{-6}	5.86×10^{-24}	negligible

By the time a vehicle has reached a 45,000-km altitude it is approximately at the outer limit of the outer radiation zone, and all counters should be counting the normal cosmic-ray intensity of about three counts per second. This is roughly two and a half hours after the vehicle has passed through the inner zone. Thus, the activity remaining in the plastic scintillator would be 0.01 count per second at 45,000 km, and there would be no appreciable systematic error due to induced radioactivity.

To verify this conclusion, the effective flux-cross-section product for this reaction was duplicated in the 710-MeV monoenergetic, external proton beam of the Berkeley 184-inch synchrocyclotron, and a 25-min bombardment was performed. The initial activity of a similar piece of lucite was found to be 32 disintegrations per sec, and the activity at the end of two and a half hours was 0.14 disintegrations per sec, which is in poor agreement with the predicted activity. No Be^{7} activity was detected. It should be noted that, even though such poor agreement with the calculations exists, this amount of activity is still negligible in comparison with the normal counting rates encountered at 10,000 and 45,000 km.

THE ACTIVATION OF A SPHERICAL ALUMINUM SHELL

In order to estimate the consequences of activating a space vehicle, a simple model must be assumed so results can be obtained from known data. The model assumed here is a space vehicle equivalent to a spherical aluminum shell

one cm thick and 31.6 cm in radius. This corresponds to a total volume of 1240 cm^3 of aluminum with very little self-absorption of the induced gamma radiation and positron-annihilation radiation.

From Table 2, in which activities are determined from Table 1 and in which dose-rates at the center of the shell are calculated on the basis of Mayneord's reciprocal dose theorem (60), it is apparent that the radiation dose contributed by activation of such a vehicle would be negligible. In fact, the order of magnitude is so small that it is safe to say that the similar activation of heavier metals such as iron and copper by spallation and fission would not contribute to the radiation dose inside a vehicle, although much higher activities would be expected (45,46,61,62).

Table 2. Point gamma-ray dose at the center of an activated spherical aluminum shell

Product nuclide	Total activity (μC)	Initial dose rate (μr/hr)(63)	Infinite time dose (μr)
Na^{24}	.00189	0.359	7.8
F^{18}	.0226	1.36	3.6
			11.4 Total

A qualitative experiment was performed using the 340-MeV proton beam of the Berkeley 184-inch synchrocyclotron. Activities observed were Na^{24}, F^{18}, C^{11}, Mg^{27} and some other unidentified species.

THE ACTIVATION OF MAMMALIAN TISSUE

Since it is expected that mammals will one day pass through the inner radiation zone (64), it is of interest to attempt to predict the consequences of induced radioactivity following a single traverse of the inner zone. Here, a consideration of the standard man may suffice. The standard man weighs 70 kg, is 170 cm tall and contains 12.6 kg of carbon, 2.10 kg of nitrogen and 45.5 kg of oxygen (65). On this basis, the total activity of C^{11}, N^{13} and O^{15} can be determined, and the infinite integral absorbed gamma-ray dose can be calculated by the method of Bush (66) by utilizing the dose-rate data of Marinelli et al. (67). These are given in Table 3.

From Table 3 it is seen that the absorbed integral dose due to tissue activation is about five gram-roentgens, and that this total-body gamma-ray dose of about 50 μr is not detectable, particularly in comparison with the expected 5 rads to be received in a lunar transfer (64,68).

Table 3. Activity and dose due to radionuclides activated in standard man

Product nuclide	Total activity (μC)	Initial dose rate (g-r/hr)	Integral infinite dose (g-r)
C^{11}	15.2	5.8	2.9
N^{13}	16.2	6.3	1.5
O^{15}	20.6	8.1	0.4

To complete the discussion, the positron doses of the induced activities must be considered. These are also found unimportant when compared with the published maximum permissible body burdens for the positron activities of C^{11} and N^{13} as in Table 4 (67).

Table 4. Comparison of total induced activity with permissible body burden of standard man

Radioisotope	Total activity (μC)	Permissible body burden (μC) (67)
C^{11}	15.2	1490
N^{13}	16.2	2420
O^{15}	20.6	not given

A 100-g sample of live tissue (a live rat) was activated under the simulated conditions of a single traverse through the inner radiation zone. Again, 710-MeV proton beam of the Berkeley synchrocyclotron was used. The resulting activity should be one seven-hundredth of that calculated for the standard man. Table 5 shows a comparison of the calculated activities with the experimental observations multiplied by 700 for each isotope. No Be^{7} activity was detected.

The striking agreement found in Table 5 suggests that the calculated values may have some validity and that it is safe to proceed on that assumption.

ACTIVATION OF PLANETARY EXPLORATION APPARATUS

Proton activation has been considered as a method for non-destructive analysis of the lunar surface (69). For this purpose a sodium-iodide scintillation detector was suggested. In a traverse through the inner radiation zone, Na^{23}, Mg^{23}, I^{126}, I^{125}, I^{124}, I^{121} and I^{120} are all formed with appreciable cross-sections (46,70,71). Among these radioisotopes, I^{126}, I^{124} and I^{121} have

Table 5. Comparison of calculated initial activity of standard man with observed initial activity of activated rat corrected for mass. No O^{15} activity was observed, as three half-lives had passed before counting began

Radioisotope	Observed initial activity (μC)	Calculated initial activity (μC)
C^{11}	12.4	15.2
N^{13}	9.2	16.2
O^{15}	----	20.6

half-lives such that they may confuse the background activity of the proposed apparatus.

This method has been abandoned in favor of a neutron-gamma inelastic scattering technique (72,73), in which the sodium-iodide crystal will be used with a 256-channel pulse-height analyzer in which pulses will be carefully selected with respect to time and magnitude to obviate background ambiguities. The same detector is proposed for cosmic-ray and background measurements, but the problem of induced radioactivity must be kept in mind, particularly if pulse-height analysis is to be performed.

An apparatus is being designed for the detection of gamma radiation due to potassium-40 activity on the lunar surface (74,75). The detector consists of a 2.75-in x 2.75-in cylindrical CsI(Tl) scintillation crystal in a sandwich of plastic scintillating material 1/8 in. thick. The purpose of the plastic is to reject the counting of cosmic ray particles by coincidence.

The total volume of the CsI portion of the detector is

$$(2.54)^3(2.75)^3(\pi/4) = 268 \text{ cm}^3$$

or, because CsI has a density of 4.510 g/cm^3, the total mass is 1,210 g of CsI. This means the detector contains 28 x 10^{23} atoms of Cs and of I.

Since, in the references cited, it is proposed to count γ-rays only in the energy range 1.36 to 1.56 MeV, only nuclear reactions yielding isotopes which emit γ-rays in this energy range (23) need be considered. These reactions are tabulated in Table 6 with their corresponding cross-sections, as available from the general literature (46,71).

The data in Table 6 are meager and the proton energy spectrum is strongly balanced toward the lower energies (see Fig. 2). Therefore the value of the cross-section at 100 MeV will be taken to hold for the total mean proton flux of 1.7 x 10^4 protons/cm^2-sec, as determined from Fig. 1. Thus, Table 7 lists the

Table 6. Proton reactions with nuclei of the scintillation detector

Proton reaction	Gamma-ray energy (MeV)	Product half life	Bombarding energy (MeV)	Reaction cross-section (mb)
$I^{127}(p,pn)I^{126}$	1.41 (ca. 1%)	13.3 d	100	126 ± 26
			170	64 ± 9
			300	53 ± 8
			480	70 ± 9
			660	51 ± 10
$I^{127}(p,p3n)I^{124}$	1.72	4.5 d	100	50 ± 7
			170	54 ± 12
			300	28 ± 10
			480	19.5 ± 4.6
			660	17.3 ± 3.0
$Cs^{133}(p,p5n)Cs^{128}$	1.50	3.8 m	100	ca. 60

cross-sections which are to be used in the determination of the activity of the crystal, along with the corresponding calculated effective flux-cross-section product. In Table 6 the reaction cross-section for the reaction $Cs^{133}(p,p5n)Cs^{128}$ at 100 MeV was approximated by interpolation of the data of reference (71).

Assuming a 14-day decay period for the isotopes during the lunar transfer, and a 25-minute activation time, as before, it is clear that the activity due to Cs^{128} will have disappeared beyond detectable limits by the time the apparatus reaches the moon. This is not true of the iodine daughters in Table 7, however. Table 8 shows the total induced activities calculated for I^{126} and I^{124} at "zero time" after activation and at the time of arrival on the moon. In view of the fact that the radioactive nuclei are within the crystal, the total self-counting efficiency should be quite high. Experience in our laboratory suggests that a photoelectric detection efficiency of 20% is not unreasonable for the energy range in question. Since the overlap of the I^{124} γ-ray photoelectric peak will be very small in the 1.36-1.56 MeV range, a two-percent photoelectric-detection efficiency is assumed for the apparatus in question. Note that the 1.41-MeV γ-ray occurs in about 1% of all disintegrations of I^{126} (23).

Induced radioactivity contributes approximately 1.1 additional counts per minute in this device. It should be noted that this is the result of a liberal calculation, and that this figure should represent a maximum self-counting rate due to induced radioactivity.

Table 7. Assumed mean cross-sections and effective flux-cross-section products for proton reactions with nuclei of the scintillation detector

Proton reaction	Assumed σ (mb)	$\overline{\phi\sigma}$ Product (protons/atoms-sec)
$I^{127}(p,pn)I^{126}$	130	221×10^{-23}
$I^{127}(p,p3n)I^{124}$	50	85×10^{-23}
$Cs^{133}(p,p5n)Cs^{128}$	60	102×10^{-23}

Thus, under the proposed experimental conditions (75), the total background counting rate will be increased by the addition of the self-counting of induced radioactivity, giving a total background counting rate of about 18 count/min against an expected minimum of 3 count/min due to lunar K^{40}. (The total background was previously estimated at ca. 17 count/min (76).

The standard deviation of the total count rate for the 10 min of proposed detection turns out to be

$$\sigma = \sqrt{(3+18)/10} = 1.45 \text{ count/min},$$

which further reduces the possibility of detecting potassium 40 on the lunar surface, unless its activity is much higher than the expected minimum.

CONCLUSIONS

The single conclusion to be derived from the foregoing discussion is that the problem of activation in space vehicles by radiation-belt protons is of little significance to space research. It appears that proton activation is neither detrimental nor useful to research to be conducted with space vehicles. It would be extremely difficult to obtain statistically useful data from activated materials retrieved after a traverse through the inner radiation zone. Nevertheless, if very sensitive detectors are to be used in vehicles weighing several hundred kilograms, corrections for proton activation may need to be considered.

DISCUSSION

This entire discussion is based on a large number of approximations. The most serious errors have probably resulted from the numerical integrations and extrapolation of satellite counting data. Also, more accurate data on the inner-belt protons have become available since the writing of this paper. Although errors may be as great as a factor of ten in some instances, the ultimate conclusion of the discussion will not be significantly altered.

Table 8. Total induced activities and self-counting rates of iodine isotopes activated in the CsI detector by the Van Allen belt protons

Iodine isotope	Initial activity	Activity at moon	Detection efficiency	Count rate at moon
I^{126}	336 dpm	164 dpm x 1%	20%	0.33 cpm
I^{124}	330 dpm	39 dpm	2%	0.78 cpm

A great deal of liberty has been taken in presenting the excitation functions. Data from various sources have often been at extremely great variance, and some excitation functions are based on meager data. The reactions were chosen mainly because of the availability of cross-section data. This implies that some reactions may have been omitted, but this is not generally the case for the parent nuclides considered, as is pointed out in the section on nuclear reaction of primary interest.

It must be added that the activation of long-lived isotopes may take on some significance if a vehicle is to spend several days in the inner radiation zone. This is an entirely different problem, but it could be investigated on the basis of this discussion. No account has been made for shielding in space vehicles, and the problem of activation during solar flares is not considered.

SUMMARY

Proton-induced radioactivity in the inner radiation belt has been analyzed from available experimental data in the literature prior to 1961 to estimate its relevance to space flight safety and instrumentation. Proton-excitation functions of carbon, nitrogen, oxygen and aluminum are presented and used to calculate induced radioactivity in a scintillation detector, an aluminum shell and in the "standard man." Planetary exploration apparatus is also considered. In these cases, induced radioactivity in the inner radiation belt has been found measurable but small. It presents little problem to space flight and exploration but can give rise to a small increase in count rate if low-background counting is performed by specialized detecting devices. In particular, proton-induced radioactivity was found to influence a detection system designed for the determination of the potassium 40 content of the lunar surface.

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Neutron-Activation Analysis for Phosphorus in a Study of Development in a Beetle Wing

James S. Beck and Thomas R. Manney

While investigating a radiation effect on wing development in the confused flour beetle (Tribolium confusum), we found it desirable to quantitate in some manner differences between normal and affected wings at different stages of development. A method was developed for reproducibly isolating the minute (<20 μg) membranous wings which, during the pupal stage, contain a population of more than half a million hypodermal cells (1). We wished to make measurements on individual wings in order to assess the variation from one animal to another, while sampling enough wings to detect small differences among different groups. Neutron-activation analysis has been established as a method of unparalleled sensitivity for many elements of biological importance (2,3), and preliminary experiments showed that we could easily measure phosphorus in these wings by this method. The occurrence of this element in both nucleic acids and lipoproteins makes it a reasonable choice as an indicator of changes in cellular state and number.

When samples of nearly any material are exposed, under identical conditions, to the same flux of neutrons, a constant fraction of the various nuclides in each sample becomes radioactive. For each element the intensity of this induced activity is proportional to the amount of the element present in the sample. The radioactivity induced in each element is unique and can often be distinguished from other radioactivities by physical or chemical means. Therefore, by activating a standard sample containing a known amount of the element of interest, together with the sample to be analyzed, one can determine the amount of a given element in the specimen.

MATERIALS AND METHODS

The procedure used in our studies was as follows: Several samples of wings and a separate, dry, weighed sample of KH_2PO_4 were exposed simultaneously for 8 hr in the Livermore Pool-Type Reactor (LPTR), which is operated at the Livermore Site of the University of California Lawrence Radiation Laboratory. The flux was about 5×10^{12} thermal neutrons cm^{-2} sec^{-1}. For the first experiment the wings were packaged in two groups in polyethylene vials, which in turn were sealed in a standard LPTR aluminum container. These wings suffered appreciable radiation damage and became somewhat brittle, making them difficult to

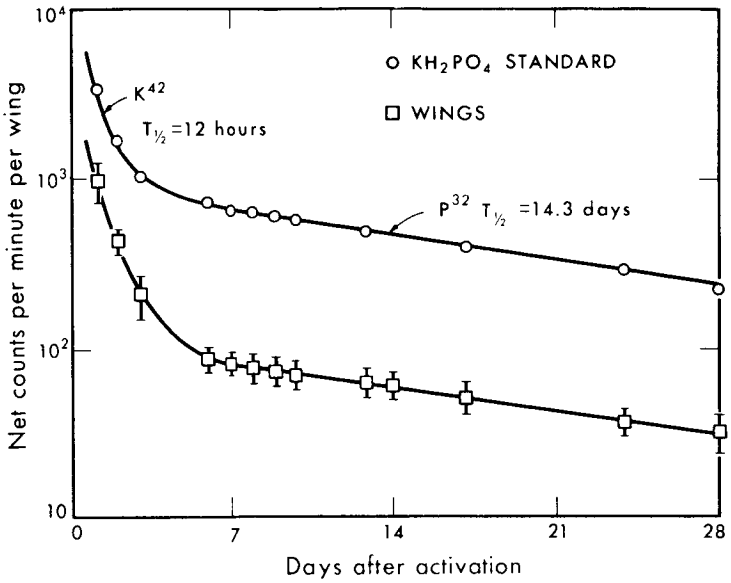

Figure 1. Radioactive decay of activated wings and standards.

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handle. We had found previously that radiation damage to thin polyethylene was reduced by maintaining good thermal contact with the coolant of the reactor. In the subsequent experiment, therefore, good thermal contact was established by packaging each group of wings in a tiny polyethylene envelope, sandwiching these envelopes between aluminum foils and stacking them in a standard LPTR container.

For counting, each wing was attached with double-faced tape to a polyethylene sample holder. Background counts were obtained from similar preparations without wings. Standards containing 0.28 μg of phosphorus were prepared in duplicate by dissolving the activated KH_2PO_4 in H_2O and evaporating 2 μl of this solution onto a holder. These were placed in an automatic sample changer and successively counted with an end window Geiger-Mueller tube. A Sr^{90} source was also counted to monitor the long term stability of the counting system. Counts and counting times were recorded by a printer, making the entire counting operation automatic.

RESULTS

In the first experiment eight wings from adults approximately 24 hr past eclosion were analyzed. The radioactivities of the eight individual wings were averaged. These averages are plotted semilogarithmically against the time after activation in Fig. 1. The vertical line at each point represents twice the observed standard deviation among the eight wings. The early counts reflect the presence of short-lived radionuclides, primarily Na^{24} (half-life 15 hr). From the 6th day to the 28th day (the time of the last count) the activity decayed with the same half-life as that of the standard, which corresponds to the 14-day half-life of P^{32}. Gamma-ray scintillation spectroscopy of a pooled sample failed to detect the presence of long-lived gamma-emitting nuclides. Comparison

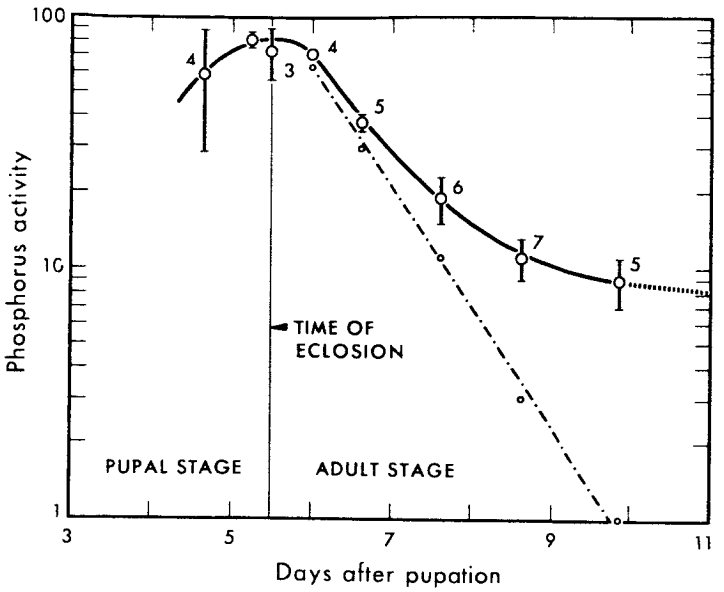

Figure 2. Phosphorus activity (arbitrary units) as a function of stage of development. Numbers of wings in each sample are shown on the graph.

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with the KH_2PO_4 standard indicated 0.03 μg of phosphorus per wing.

In another experiment we measured the amount of phosphorus in imaginal (final) wings removed from the animal at various times during pupal and early adult life. Dissection of imaginal membranous wings from within the pupal cuticle is difficult at earlier stages, and larger sampling errors result. However, useful data were obtained, even with small numbers of wings. Counting was begun 7 days after the activation and continued until each wing had been counted twice. The interval between the two counts of any given sample was 5 days. The expected 14-day half-life was verified for each sample, and the counts were corrected for decay to an arbitrary time to make comparison possible. In Fig. 2 these values are plotted semilogarithmically as phosphorus activity per wing against the time of development at which the wing was sampled (solid curve). Assuming that the amount of phosphorus in wings one day after eclosion was the same as in the first experiment, the latest sample, taken 10 days after pupation, contained 0.005 μg of phosphorus per wing. The standard deviation for the five wings of this sample is 0.001 μg. This includes both the variation among the wings and the variation of the counts. The standard deviation of the counting of this smallest quantity (2×10^3 net counts in 1 hr) is only about 2%.

DISCUSSION

Observations by light and electron microscopy have revealed that after eclosion the hypodermal cells are lost from the wing. Failure to observe cell fragments suggests that this process is a transfer of whole cells from the wing. Accordingly, the observed decline in the phosphorus would reflect this loss. The curve seems to approach a constant value which would represent the amount of phosphorus in the wing structure exclusive of that in the hypodermal cells. When

our estimate of this value, obtained by extrapolation (dotted line of Fig. 2), is subtracted from the data, an exponential curve results (broken line of Fig. 2). Our interpretation of this result is that the cells leave the wing at a rate proportional to the number in the wing, i.e., randomly. This is in striking contrast with the pre-eclosion state of the hypodermal cell population (1). The cells were then a fixed population precisely synchronized in a regulated sequence of steps in differentiation and morphogenesis.

SUMMARY

Neutron activation analysis was used to measure phosphorus in individual beetle wings during pupal and early adult stages. By counting neutron-induced P^{32} radioactivity it was possible to measure 0.005 ± 0.001 μg of phosphorus. The phosphorus content of the wings rises to a maximum at eclosion and subsequently decreases with loss of cells.

ACKNOWLEDGEMENTS

This study is based on work performed under contracts with the U.S. Atomic Energy Commission.

We are grateful to Dr. A. Kirschbaum and Mr. E. Hill for the use of the reactor and counting facilities and to Mr. R. Padden for technical assistance.

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Normal Iron Absorption in Man

Hiroshi Saito, Thornton Sargent, Howard G. Parker and John H. Lawrence

The whole-body counter, which can detect the retention of small amounts of γ-emitting radionuclides (1), has simplified the study of iron absorption. It has the advantage of being more accurate than other devices because absorption of iron is usually so small that indirect methods may produce considerable error. Furthermore, the high sensitivity of the low-background whole-body counter permits the use of a small quantity of radioiron, much less than the maximal permissible body burden.

Lushbaugh et al. (2) and Van Hoek et al. (3) have studied iron absorption with whole-body liquid-scintillation counters, but the number of whole-body counters available for clinical uses is still limited at the present time. Therefore, the method presented in this study is believed to offer considerable advantages, especially in clinical application.

MATERIALS AND METHODS

Twelve normal volunteer males, over 19 years of age, were subjects of study. A history of previous condition of health was recorded for each, and blood tests were performed in order to exclude subjects showing abnormal conditions which might affect iron metabolism.

A low-background whole-body counter, having six-inch steel walls and a nine and three-eighths by four-inch crystal of NaI(Tl) with a one-hundred-channel pulse-height analyzer, was used. A detailed report on the counter was presented by Sargent at the IAEA Symposium in 1961 (4). A well-type scintillation counter with single-channel analyzer was used for Fe^{59} labeled blood sample counting. The subjects were placed for counting on a couch with a radius of curvature of one meter with the crystal at the center, using the "one meter arc" geometry.

Red cell volume determination was made by incubating red cells in vitro with 20 μC P^{32}, washed three times with serum and then returned to the subject's vein, the incubum containing approximately 2 μC of P^{32}. Using an indwelling needle, a blood sample of 1 ml was withdrawn every 15 min for 60 min. Blood samples were placed on aluminum planchettes and counted with a low-background thin-window G.M. counter. On each subject the following hematological examinations were performed: RBC count, reticulocyte count, Wintrobe hematocrit, hemoglobin concentration, serum iron, unsaturated iron-binding capacity.

The subject reported for his first day without breakfast, and a 20-minute

background was obtained of the subject in the whole-body counter. The oral dose of radioiron was counted in a vial in the whole-body counter. The radioiron was transferred to a plastic cup and the carrier iron added. The subject received about 5 cc of the liquid containing 2 μC of radioactive tracer in the form of ferric chloride. In five subjects the dose was varied in successive tests of carrier iron: no carrier (less than 4 μg), 4 mg, 30 mg, and 60 mg in the form of ferrous sulfate solution (Feosol elixir). In seven subjects, four successive tests of 4 mg carrier each were used to measure the reliability of the test. Each subject drank the dose followed by three water rinses of the cup. He was counted (in the one-meter-arc geometry) for five minutes just after taking the iron dose. Subjects were not allowed to eat for two hours after taking the dose. Each subject was counted every other day after his first dose for two weeks or longer to determine the rate of elimination. Counts were made until there was no further excretion, i.e., until the whole-body count remained essentially constant.

Two weeks after the oral dose 3 cc of blood were taken in order to obtain percent utilization of the absorbed radioiron for hemoglobin formation. At intervals of two weeks, subjects received the successive doses, without further observation of elimination curves. Percent absorption was calculated from the whole-body count obtained two weeks after the dose, subtracting the background and activity due to the previously absorbed radioiron. The equivalent of 100% was calculated from a measurement of whole-body activity two weeks after an intravenous dose instead of using a phantom.

After four absorption-test doses, the subject received 5 μC of Fe^{59} intravenously in the form of ferric chloride without carrier and was counted immediately in the whole-body counter for 5 min. This was followed by five-minute counts once a day for the first week after the intravenous dose, and three times during the second week. The injected Fe^{59} was not incubated with plasma which, according to Hallberg (5), gives essentially the same results as when it is incubated. Using an indwelling needle, a 3-cc blood sample was taken every 15 min for 60 min. For plasma volume determination 1 cc of plasma was also counted with a well counter.

Two weeks after the intravenous injection, a 3-cc blood sample was taken to obtain a percent utilization figure. This was for comparison with the percent utilization calculated from the blood radioactivity following the first oral dose. Geometry correction was done from naturally sedimented RBC to well mixed blood.

Serial whole-body counts were done after the intravenous radioiron injection to observe whether any changes occurred in the whole-body count due to the movement of radioiron from serum to bone marrow to peripheral red cells.

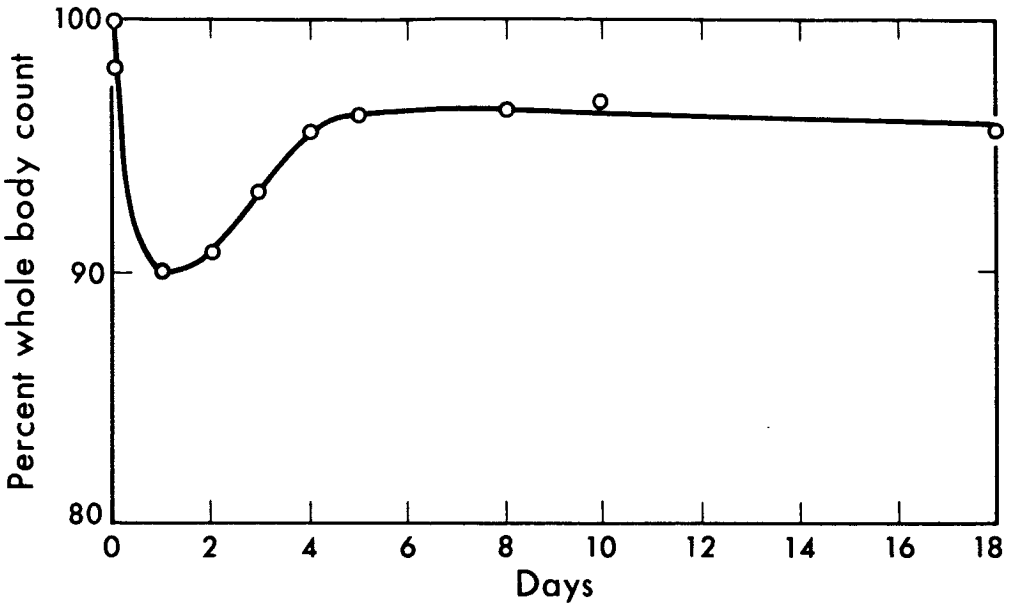

Figure 1. Change of whole body count after intravenous injection of Fe^{59}.

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absorption of future patients, thereby eliminating the need for an intravenous dose.

After the intravenous radioiron dose, a decrease of whole-body count from 100% to 90% at 24 hr was observed, followed by a return to a plateau of 96.5% thereafter, as shown in Fig. 1.

The decrease of whole-body count after intravenous injection corresponds with the disappearance of radioiron from the serum. The lowest count was obtained when most of the radioiron was fixed in the bone marrow and in storage organs. This change of geometry (radioiron distribution) caused the decrease of whole-body count. Thereafter radioiron is released from the bone marrow into the peripheral blood stream as the red cell precursors mature. The whole-body activity, however, did not return to the initial 100%; the average of nine subjects was 96.5% at two weeks. (This is taken as 100% dose for absorption at two weeks after oral dose.) This may imply that some of the radioiron is in storage in the liver, spleen, etc., and that the average distance of radioiron from the crystal in these organs is greater than when radioiron is in peripheral circulation. The reason why the whole-body count did not return to 100% is not entirely clear, but the count at two weeks can be used as a reliable basis for determining iron absorption.

Most of the unabsorbed radioiron was eliminated during the first five

Table 1.

	Whole body activity immediately after oral dose; vial activity before oral dose					Whole body activity immediately after intravenous injection; syringe activity before injection
	Experiments					
	1	2	3	4	Mean	
C.D.	1.29	1.29	1.46	1.30	1.34	1.10
R.G.	1.35	1.34	1.30	1.34	1.33	1.09
K.K.	1.41	1.34	1.17	1.39	1.33	1.15
R.M.	1.30	1.41	1.32	1.30	1.33	1.15
M.M.	1.37	1.39	1.36	1.24	1.34	1.15
H.P.	1.40	1.23	1.41	1.44	1.37	1.12
J.T.	1.48	1.37	1.46	1.33	1.41	*
T.Y.	1.42	1.36	1.45	1.42	1.41	1.10
H.W.	1.29	1.23	1.19	1.24	1.24	1.14
F.W.	1.39	1.75	--	1.71	1.62	1.10
C.R.	1.28	1.26	1.29	1.20	1.26	1.12
B.T.	1.26	1.29	1.26	1.26	1.27	1.12
	Mean				1.35 ± 0.09	1.12 ± 0.01
	Range				1.19 - 1.75	1.00 - 1.15

*Subcutaneous injection

Subjects were not permitted to stay at altitudes of more than 5,000 feet for more than five days during the first three months of the absorption study, nor to donate blood throughout the period of study.

RESULTS AND DISCUSSION

The ratio of the whole-body count of an oral dose immediately after ingestion to the count of the vial alone on the one-meter arc was not constant even in the same subject (comparing four oral doses). On the other hand, the ratio of the whole-body count of the intravenous dose in the circulating blood immediately after the injection to the count of the syringe (geometry of a dose in a syringe is the same as in a vial, i.e., on the one-meter arc) was almost constant: 1.12 ± 0.01. These results are shown in Table 1.

Therefore, the count from the vial before ingestion was multiplied by the ratio: intravenous count in subject/syringe count to yield a count equivalent to 100% absorption. However, the whole-body count two weeks after the intravenous dose was 96.5% of the count just after intravenous injection, as will be discussed below. Therefore the ratio 1.12 x 0.965 = 1.08 can be applied to the count of the vial on the arc before ingestion to calculate the percent

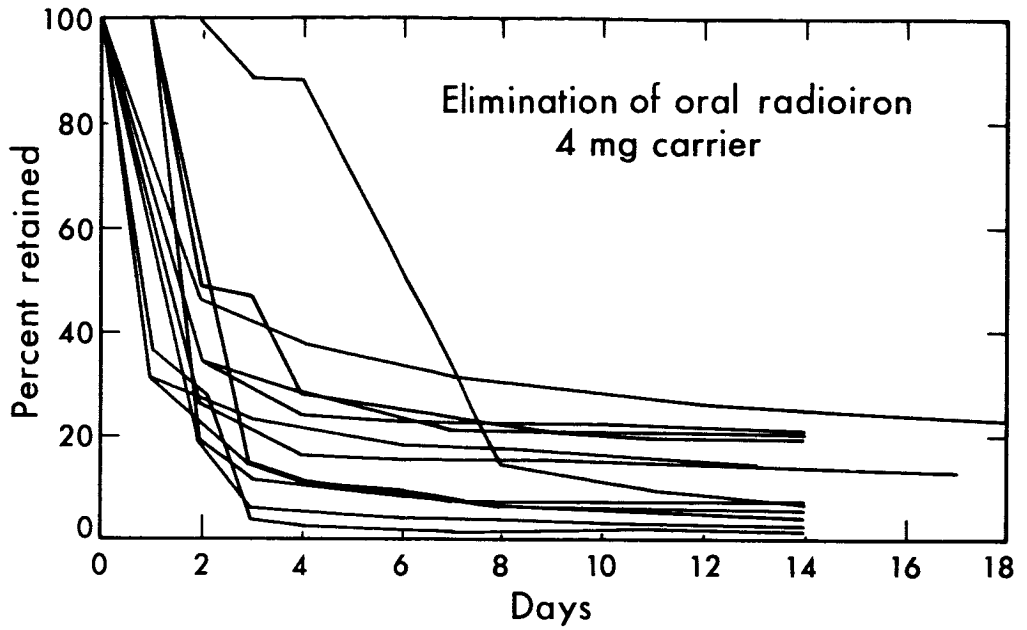

Figure 2. Elimination of unabsorbed radioiron from the intestinal lumen after oral dose with 4 mg of carrier.

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days, except for one constipated subject. Two weeks or more elapsed before it was eliminated completely. A flat elimination curve at two weeks indicates constant retention of radioiron in the body. Fig. 2 shows the variability of the elimination curves; the retention of one subject showed about 90% decrease of whole-body count after only one defecation, but another subject showed none after one defecation. About 76% of unabsorbed iron was excreted during the first five days, but about 23% was excreted thereafter within a period of two weeks or more. Elimination of unabsorbed radioiron depends on the frequency and volume of defecation, but defecation was not always accompanied by a decrease of whole-body counts.

Fecal assay as done by Chodos et al. (6), Dubach et al. (7), Smith et al. (8), and Bonnet et al. (9) is difficult to conduct for a longer period, and the small amounts excreted after five days are often difficult to detect. This is probably the reason why fecal methods have sometimes given higher values of percent absorption of iron. Bothwell (10) found higher values by the fecal method than by the double isotope method.

Normal subjects showed a range of 1.3% to 22.8% absorption with 4 mg of carrier. The average of twelve subjects was 11.4 $\pm$ 2.3. The average of seven subjects with a 4 mg dose repeated four times was 9.1 $\pm$ 1.3 (Table 2).

The correlation between percent absorption and reticulocyte count is

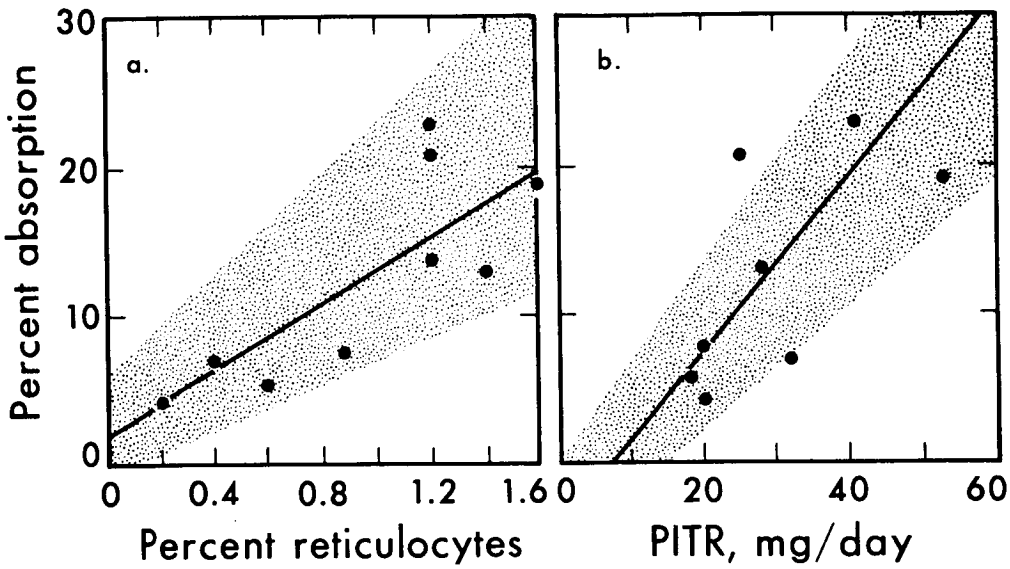

Figure 3a. Correlation between percent absorption and reticulocyte count.

Figure 3b. Correlation between percent absorption and plasma iron turnover rate.

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shown in Fig. 3a. The range of normal values of iron absorption is wide, but when it is compared to the reticulocyte count, the normal absorption-reticulocyte range becomes much more sharply defined, as indicated by shaded area in Fig. 3a. This narrower range will be very important in distinguishing cases of suspected increased iron absorption (as in iron deficiency or hemochromatosis), or decreased absorption (as in gastric resection or malabsorption syndromes).

One subject, who received a 4 mg dose repeatedly, showed a gradual decrease of percent absorption from 22.8 to 7.0 while his reticulocyte count also decreased from 1.2 to 0.2 during an eight-week period. Although the correlation between reticulocyte count and absorption was clear, the reason for the gradual change in this subject was not discovered.

The correlation between percent absorption and reticulocyte count was anticipated, since Stewart et al. with dogs (11), Washino (12), and Saylor and Finch with rats (13) reported that animals with a hemolytic condition showed high reticulocyte counts and high absorption in spite of sufficient body iron. The correlation found here (Table 3) may suggest the existence of small hemolysis in some normal subjects. Iron retention studies on subjects with high absorption and high reticulocyte counts showed that their iron loss was not abnormally high (as will be reported later). It was thought, therefore, that a mild hemolysis was a more likely cause of the elevated reticulocyte count.

Table 2. Percent absorption with 4 mg carrier

R.G.	22.8	19.8	7.3	7.0	14.2 ± 4.2
K.K.	5.6	4.3	6.9	6.8	5.9 ± 0.6
M.M.	6.9	11.4	3.3	1.9	5.8 ± 2.1
T.Y.	19.1	18.6	15.6	13.8	16.8 ± 4.3
F.W.	1.3	1.3	1.5	2.3	1.6 ± 0.3
C.R.	2.7	3.3	1.7	8.1	4.0 ± 1.1
B.T.	19.3	6.4	20.3	16.7	15.7 ± 3.2
Mean					9.1 ± 1.3

A correlation between percent iron absorption and PITR was also found, as shown in Fig. 3b. These correlations imply that the degree of iron absorption is directly related to the level of erythropoietic activity.

Percent absorption decreased with increasing carrier dose up to 30 mg, and it was less scattered at larger carrier doses (30 to 60 mg), as shown in Fig. 4a and Table 4. The total amount of iron absorbed, however, increased up to 60 mg, the maximal dose given, in proportion to the level of carrier iron, as shown in Fig. 4b.

The hypothesis of mucosal block proposed by Hahn (14) and Granick (15) could partly explain the mechanism of iron absorption on the basis of the existence and the characteristics of ferritin in the mucosa. However, Gilman and Ivy (16) showed hemosiderin was formed in the process of iron absorption, and Heilmeyer et al. (17) expressed doubts about the existence of the automatic control of the absorption of iron by ferritin. If percent absorption is considered, there appears in the present study to be a relative blockage of iron absorption from 0 (less than 4 μg) to 30 mg, as shown in Fig. 4a. A linear increase of the total amount of iron absorbed with amount of carrier given is in agreement with the results obtained by Smith et al. (8). This incomplete blockage would depend on the nature of hemosiderin formation in the intestinal mucosa as reported by Saito (18,19).

Both the intravenously injected iron and iron absorbed from the oral dose showed good percent utilization* (average 91.3 ± 2.2 and 90.2 ± 0.6). Although there was a two-month interval between the first oral dose and the intravenous injection, there was no significant difference between them.

$$*\%\ \text{utilization after IV injection} = \frac{Fe^{59}\ \text{appearing in the erythrocytes}}{\text{total } Fe^{59}\ \text{injected}}$$

$$\%\ \text{utilization after oral} = \frac{Fe^{59}\ \text{appearing in the erythrocytes}}{Fe^{59}\ \text{absorbed, calculated from whole body counts}}$$

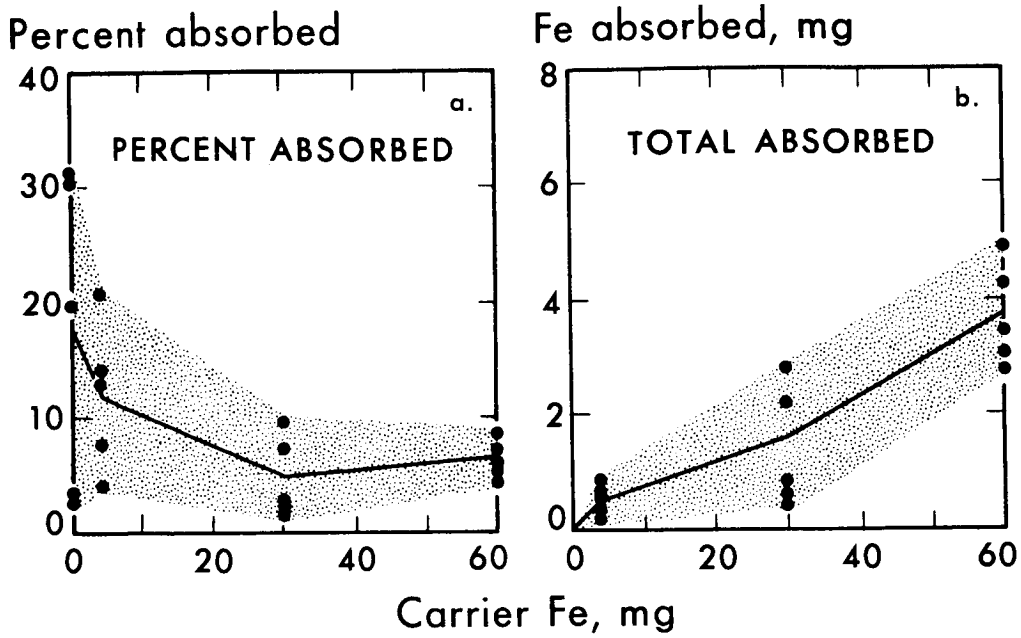

Figure 4a. Relationship between carrier iron and percent absorption. (Relative blockage of percent absorption from 0, less than 4μg, to 30 mg.)

Figure 4b. Relationship between carrier iron and iron absorption. (The total amount of iron absorbed increased up to 60 mg.)

In cases of low absorption the fraction of utilized radioiron in the 3-cc whole blood gave so few counts over background that it required long counting times to obtain good accuracy.

Both absorbed and intravenously-injected radioiron showed good percent utilization in spite of the different routes followed, as shown in Table 5. Although absorbed radioiron passes through the portal vein, fixation of iron in stores before incorporation of radioiron into the red-cell precursors would not be expected to occur because iron is fixed rapidly in the liver, and it is also released rapidly, i.e., the turnover is rapid (12,18,19). Saylor and Finch (13), in using the double isotope method to study iron absorption in rats, assumed that the percent utilization of radioiron is the same whether it enters the circulation via a peripheral vein or via intestinal vessels. The results of the present study support this assumption and also demonstrate the value of administering a single isotope twice, orally and intravenously, by which oral absorption can be measured reliably without a whole-body counter. Such a test may be employed for clinical purposes without any technical difficulty if the hematopoietic condition of the patient stays constant during the test period, i.e., about four weeks or less. Menstruating females may be somewhat more complicated to study. In the double isotope method, the preparation for the counting of the Fe^{55} X ray is not simple, although Bothwell *et al.* (10) and Hallberg and Sövell

Table 3. Correlations between % absorption, % reticulocytes and plasma iron turnover rate

Name	% Absorption	% Reticulocytes	PITR mg/day
C.D.	13.0	1.4	20.8
R.G.	22.8	1.2	40.9
K.K.	5.6	0.6	19.3
R.M.	7.5	0.9	19.6
M.M.	6.9	0.4	32.0
H.P.	4.0	0.2	20.1
J.T.	13.7	1.2	*
T.Y.	19.1	1.6	52.8
H.W.	20.7	1.2	25.3
F.W.	1.5	0.2	73.0
C.R.	1.7	0.1	45.9
B.T.	20.3	0.8	25.5

*Subcutaneous injection.

(5) demonstrated its usefulness. With the single isotope technique such time-consuming preparations are not required.

The formula to be used is as follows:

$$\% \text{ absorption} = \frac{Fe^{59} \text{ absorbed}}{Fe^{59} \text{ oral dose}} \times 100 = \frac{A}{O} \times 100.$$

Since the percent utilization is the same via the gastrointestinal tract or intravenously,

$$\frac{U_o V}{A} = \frac{U_i V}{I} \qquad \text{or } A = \frac{U_o I}{U_i}$$

Substituting for A, $\% \text{ absorption} = \frac{U_o I}{U_i O} \times 100$

$A = Fe^{59}$ absorbed after oral dose, cpm.

O = orally-given total activity, cpm.

I = injected total activity, cpm.

U_o = activity counts/min/cc in whole blood (after oral dose).

U = activity counts/min/cc in whole blood (after 4 injections).

$U_i = U - U_o$; activity counts/min/cc which appears in whole blood from intravenous dose only.

V = blood volume in cc; this cancels out in the formula.

All samples are of the same volume, are hemolyzed to prevent settling and thus changing the geometry of the sample, and counts are decay corrected.

Table 6 shows the comparison of percent absorption figures obtained by the above method and whole-body counting.

Table 4. Percent absorption with varied carrier doses

	0 mg	4 mg	30 mg	60 mg
C.D.	19.8	13.0	1.1	7.9
R.M.	2.5	7.6	7.1	5.0
H.P.	31.0	4.0	1.7	4.5
J.T.	30.9	13.7	9.0	6.9
H.W.	3.2	20.7	2.3	5.6
Mean	17.5 ± 5.3	11.8 ± 2.9	4.2 ± 1.6	6.0 ± 0.6

Table 5. Percent iron utilization

Name	Oral	Intravenous
C.D.	83.5	89.2
R.G.	85.6	98.3
K.K.	89.3	94.7
R.M.	106.6	85.7
M.M.	89.8	96.3
H.P.	102.5	95.4
J.T.	86.8	*
T.Y.	89.8	87.9
H.W.	88.2	83.1
B.T.	82.1	90.6
F.W.	99.1	88.9
C.R.	92.3	82.0
Mean	91.3 ± 2.2	90.2 ± 0.6

Table 6. Percent iron absorption

Name	Calculated from blood sample	Whole body counter
C.D.	11.1	13.0
R.G.	19.7	22.8
K.K.	5.3	5.6
R.M.	8.9	7.5
M.M.	5.7	6.9
H.P.	4.3	4.0
J.T.	*	13.7
T.Y.	20.0	19.1
H.W.	22.0	20.7

*Subcutaneous injection.

This method although useful is nevertheless not as convenient as a whole-body counter which does not require blood sampling other than for the initial hematologic tests. Hematologic and miscellaneous data of normal subjects are shown in Table 7.

SUMMARY

Oral iron absorption was studied in normal male human volunteers using a whole-body counter. An intravenous dose served as the equivalent of 100% oral absorption, and also served as correction for geometry in each subject. The following findings resulted:

1. Elimination of unabsorbed radioiron from the intestine was 76% within five days and virtually complete after two weeks.

2. Normal subjects showed a range of 1.3% to 22.8% absorption with 4 mg of carrier.

3. The normal variation of the iron absorption was much larger when the carrier dose was small, i.e., carrier free (less than 4 μg) and 4 mg. Variability decreased with increased carrier doses of 30 mg and 60 mg.

4. Total amount of iron absorbed increased in proportion to the increase of iron dose up to the highest dose of 60 mg.

5. Correlations between reticulocyte count, plasma iron turnover rate, and percent absorption were found, relationships which yield a range of normal values of absorption within narrower limits than absorption alone.

6. After intravenous injection of radioiron, a decrease of whole-body count to 90%, followed by an increase up to 96.5%, was observed; the decrease was greatest at 24 hr after injection when almost all of the radioiron was in the bone marrow. The decrease of whole-body activity is due to the disappearance of radioiron from the serum into the marrow, and the subsequent increase of whole-body activity is due to the appearance of labeled red cells in peripheral blood circulation.

7. No significant difference in percent utilization was noted between iron entering the circulating blood by injection or by absorption from the intestine. Therefore, if the hematopoietic condition of a patient does not change during the period of study, percent absorption can be calculated by the activity appearing in the red cells ten to fourteen days after oral administration and ten to fourteen days after intravenous injection. Because of its simplicity this method should be useful clinically.

ACKNOWLEDGEMENTS

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Table 7.

Name	Age	Wt kg	Height cm	RBC million	Ht %	Hb g	SI µg/dl	UIBC µg/dl	TIBC µg/dl	% Sat.	RCV cc	PV cc	BV cc	PITR
C.D.	43	81	173	537	46.0	15.0	68	422	490	13.9	2129	2571	4700	20.8
R.G.	40	87	180	572	50.0	16.5	114	374	488	23.4	2487	2586	5070	40.9
K.K.	27	51	158	518	46.0	15.3	110	289	397	27.7	1350	2317	3667	19.3
R.M.	26	70	177	535	47.0	15.0	75	389	464	16.4	2109	2516	4619	19.6
M.M.	35	62	160	495	48.5	16.0	136	334	470	28.9	1935	2550	4485	32.0
H.P.	35	79	171	501	45.5	14.8	85	345	430	19.8	2251	2413	4664	20.1
J.T.	35	73	170	493	44.5	15.8	113	390	503	22.5	1889	2365	4254	*
T.Y.	19	54	171	503	48.0	15.2	150	363	513	29.2	1773	2475	4248	52.8
H.W.	32	68	180	502	47.5	15.3	101	205	366	27.6	1836	2559	4395	25.3
F.W.	24	83	182	530	46.0	15.8	89	165	254	35.0	2727	3610	6337	73.0
B.T.	31	77	191	543	47.5	15.8	107	206	313	34.2	1927	2814	4941	25.5
C.R.	29	74	170	519	45.0	15.2	144	155	299	48.2	2053	3050	5103	45.9

*subcutaneous injection

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Whole-Body Iron Loss in Man

Hiroshi Saito, Thornton Sargent III, Howard G. Parker and John H. Lawrence

The recent development of whole-body counters has made possible a more accurate approach to determination of the rate of iron loss in man, provided the radioiron-loss rate can be assumed to represent the total-body iron-loss rate.

A previous iron-loss study by Stevens and co-workers (1) showed that the decrease of total carcass-radioiron activity in mice closely approximated the decrease of activity in large iron compartments. If a similar decrease occurs in the human body, the rate of total-body iron loss can be obtained from the rate of radioiron loss. However, this assumption is an oversimplification, as will be discussed later.

MATERIALS AND METHODS

Eight normal male human subjects, 19 to 43 years of age, were used in the present study. A history of previous health was taken from each, and blood tests were performed to exclude subjects with abnormalities of iron metabolism. A low-background whole-body counter, having a 9 x 4-inch crystal of NaI(Tl) with a 100-channel pulse-height analyzer, was used. The subjects were placed for counting on a special couch having a radius of curvature of one meter with the crystal at the center and using the "one-meter-arc" geometry. The radioiron was administered intravenously as Fe^{59} citrate at a specific activity of 10 to 20 mC/mg. Each subject received a dose of 5 μC, injected without incubation with plasma.

RESULTS AND DISCUSSION

Immediately after injection, all of the radioiron is in the circulating plasma, but after 24 hr most of the radioiron has been taken up by red-cell precursors in bone marrow. It is then released gradually into peripheral blood over a period of days. In our study the whole-body count of a subject immediately after injection was related to the counts on the succeeding 14 days as shown in Fig. 1a. At the time the radioiron in marrow was maximal, approximately one day after injection, the whole-body count fell to 90% of the initial value, thereafter slowly rising again as newly labeled red cells were released to the circulating blood. This transient decrease of whole-body count can thus be

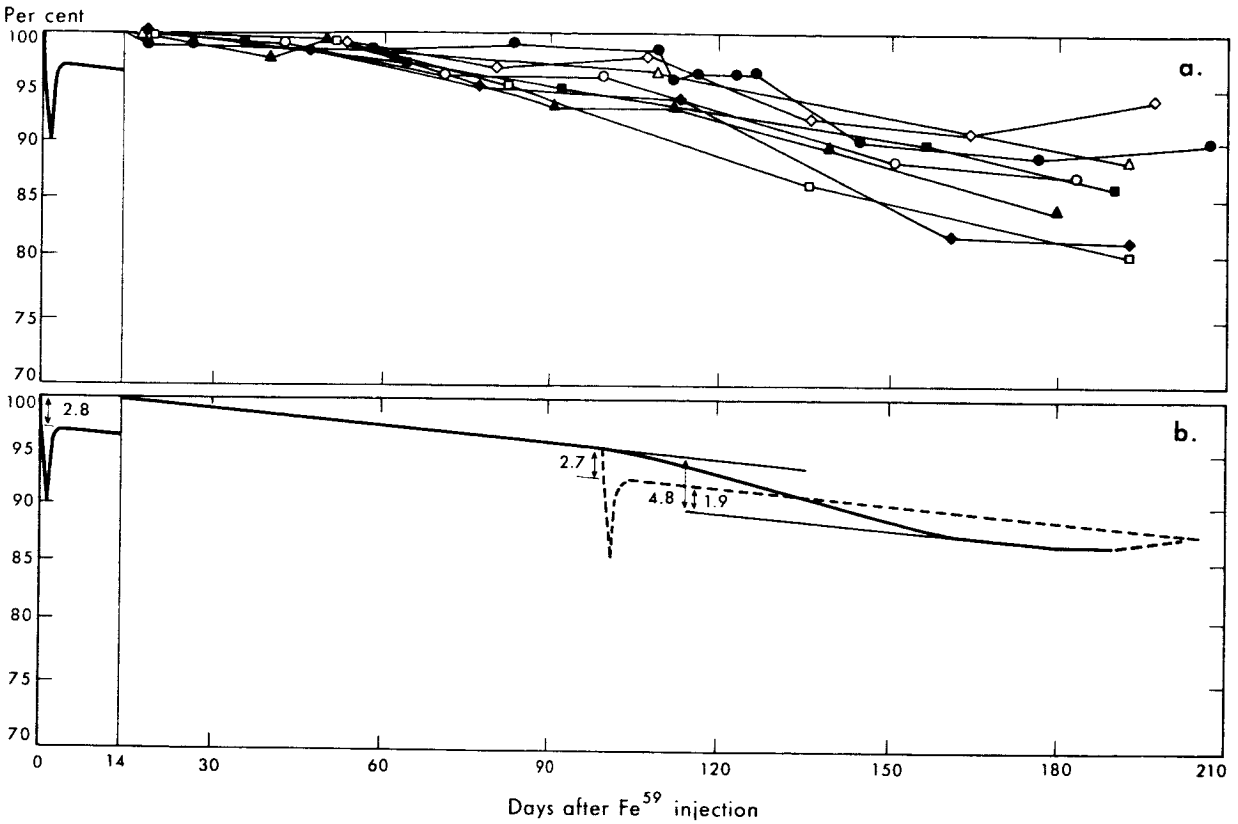

Figure 1a. Whole-body radioiron retention of the eight normal subjects, normalized to 100% at day 14.

Figure 1b. Average radioiron retention curve obtained from the curves of each of the eight normal subjects. Again it is normalized to 100% at 14 days, and in both figures the change during the first 14 days is shown as an average.
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attributed to the change of localization of radioiron. For the purposes of calculation of subsequent loss, the whole-body count at 14 days was taken as 100%.

The count returned to an average of 96.5% after two weeks, and extrapolation of the subsequent loss curve to zero time gives 97.2% on the day of injection, not 100%. This might be attributed to the following: 1. storage of some of the Fe^{59} in bone marrow and thus greater attenuation of gamma rays; 2. loss of some of the radioiron from plasma into urine before uptake into red-cell precursors. However, in one subject urine was collected for the first 24 hr after injection and only 0.2% of the isotope was found in the urine, which seems an insufficient amount to account for all of the 2.8% difference, as in 2. above. (A hemochromatotic patient who was injected with 40 μC of radioiron after incubation with serum also excreted 0.2% in the urine during the first 24 hr.) Furthermore, Dubach, Moore and Callender reported such a small amount of iron loss in feces and sweat (3) that it cannot account for this 2.8% difference. Therefore, this cannot represent solely the loss of radioiron from the body, but is probably largely the result of a change of distribution of radioiron in the body.

The data for radioiron loss of the eight subjects are shown in Fig. 1a. It is seen that the rate of radioiron loss does not vary widely among individuals for the first 100 days. The average loss during this period was 1.6 ± 0.3% per month. Average hemoglobin iron calculated from the subjects was 2,500 mg (0.334% of hemoglobin as iron by weight), and total miscible-tissue iron was taken as 490 mg (2). Therefore, total miscible iron was taken as 2,990 mg. This represents average total-body iron loss of 1.5 ± 0.3 mg per day. This daily loss is larger than previously supposed from balance study measurements (3,4). It is also larger than the maximum loss calculated by Finch on the basis of red-cell radioiron loss, which is 0.61 mg per day for normal males.

Recently Price and co-workers, using a whole-body counter, reported a higher rate of loss in three patients with normal iron metabolism (0.103 - 0.182% per day) (5) than obtained in this study (0.05% per day).

Average daily dietary iron intake is known to be about 15 mg, and the average absorption of a 4 mg dose of ferrous iron in fasting subjects tested was 9% (6). Thus the daily intake would be 1.4 mg per day, which is in reasonable agreement with the loss figures, since total-body iron is kept constant by the balance of absorption and loss of iron. However, absorption of radioiron from labeled foods has been reported to be lower than iron given while fasting (7).

At about 100 days, the loss curves in the subjects tested changed markedly, and this period of time coincides with the known life span of human red cells. The initially injected radioiron labeled essentially a single generation of red cells. When these began to die this radioiron was reutilized by the red-cell precursors in bone marrow. The percent utilization of the injected radioiron dose after 14 days was obtained for each subject by counting radioiron appearing in red cells which was found to average 90%. The initial radioiron injection can be considered as an idealized situation of the simultaneous death of one generation of labeled red cells, causing the sudden appearance of Fe^{59} in the circulating serum iron.

This same situation occurs again approximately 100 days later, except that the death of the red cells takes place with a wider time distribution, over a period up to 60 days (8).

We should expect, therefore, that the whole-body counts would again occur as in Fig. 1a, except that the spread in time of death of cells would tend to make the dip wider and shallower. The 100% decrease in peripheral-blood radioiron count 24 hr after intravenous injection can be compared with a decrease to 90% of the whole-body count, a decrease of one-tenth. Since the peripheral-blood radioiron count decreases by approximately 20% during the dying period of

the first generation of labeled red cells (8), this would be reflected as one-tenth of 20%, or 2%, decrease in whole-body counts over a period of 60 days. This 2% would thus be the expected decrease in whole-body counts due to recycling of radioiron after the death of the first generation of red cells.

Since the average utilization of radioiron was 90%, this means that about 10% was kept in stores, and this redistribution appears to account for the decrease of the whole-body count from 100% immediately after injection to 96.5% 14 days later. This same effect should occur again 100 days later. Although the capacity for utilization should be the same at 100 days in these normal subjects, storage partition should result in relatively less radioiron appearing in the second generation of red cells. This kind of step-wise decrease of whole-body activity is expected to occur until the radioiron in the body is uniformly mixed, which may take about a year (2).

In Fig. 1b, the solid line represents the average of the whole-body counts of all subjects, including the fall and return immediately after injection. The dashed curve beginning at 100 days is the same as the curve of the first 14 days after injection and represents the idealized situation if all the red cells had died on day 100.

After day 100, whole-body counts were 95.5% of the 14 days after injection. The entry of some radioiron recovered from the red cells into stores, and by a small amount of loss, would produce the 2.8% fall in whole-body count if it occurred suddenly as mentioned previously; $2.8 \times 0.955 = 2.7\%$ on day 100. From 160 to 190 days, the average loss curve is closely parallel to that before 100 days, and the decrease from the initial curve to the parallel second curve is 4.8%. This 4.8% decrease can be explained as follows:

The expected 2.0% decrease, over the period of labeled red-cell death from 100 to 160 days, will be $2.0\% \times 0.955 = 1.9\%$; the above mentioned 2.7% decrease due to redistribution is also expected. Adding these, $2.7\% + 1.9\% = 4.6\%$, which means that 4.6% decrease is expected, and the 0.2% difference from the experimental value of 4.8% may be due to the inefficiency of reutilization or due to error of counting. There seems to be a tendency for the 1.9% decrease to disappear after 190 days, while the 2.7% decrease would be expected to remain.

It can be noted in Fig. 1b that the dashed line intersects the experimental-average-loss curve at 134 days. If a mean hemoglobinization time of 4 days is subtracted from this, the 130 days obtained is in reasonable agreement with accepted values of mean erythron life span. This intersection point might be considered to correspond to the inflection point of a survival curve of the first generation of labeled red cells.

Therefore, a decrease of whole-body count after 100 days does not necessarily mean a larger loss rate of radioiron from the body. It could be assumed that the loss rate of whole-body iron is represented by the loss rate during the first 100 days, and that this will be the minimal value of normal iron loss.

Price and co-workers (4) have reported that no decrease of total red-cell activity was observed until 100 days after intravenous radioiron injection. Therefore, they concluded that the decrease of whole-body radioiron activity until 100 days is a result of the loss of nonhemoglobin iron, but it is difficult to reconcile this finding with the loss measured by whole-body counting. In our study, about one-tenth of the radioiron administered was present in miscible-tissue iron at 14 days after the intravenous injection of radioiron.

Finch and Loden (2) found that miscible-tissue iron ranged from 377 to 600 mg. If we take 490 mg as an average and apply the average daily radioiron loss rate of 0.05%, miscible-tissue iron loss will be 490 mg $\times \frac{0.05}{100} \times \frac{1}{0.1} = 2.5$ mg per day. This is too high a value to be lost, because it is more than absorbed. Using Price's value (5) of 0.136% per day, the loss becomes 6.8 mg per day. If whole-body radioiron loss rate represents the red-cell radioiron loss rate, and if we take red-cell iron as 2,500 mg, then 2,500 mg $\times \frac{0.05}{100} \times \frac{1}{0.9} = 1.4$ mg per day, or about 2.8 ml loss of blood daily. This 2.8 ml of blood loss seems too high a value for a normal person. Therefore, it seems more likely that radioiron loss occurs both from red cells and from miscible tissues, as observed by Stevens and co-workers in animal experiments (1).

By continuing the counting times of these labeled subjects until several generations of red cells have passed, the radioiron should mix more uniformly with all of the body iron, and radioiron loss might then be expected to reflect more closely the total-body iron loss. On the other hand, continuing intake of nonlabeled iron would lower the specific activity of the red-cell iron, since 90% of newly absorbed iron goes to red cells. This suggests that perhaps the most accurate way to measure total-body iron loss would be to label a subject as uniformly as possible by administering the label at intervals for several months, and then to count him for a year or more after completion of labeling.

SUMMARY

Whole-body iron loss was measured with a low-background whole-body counter using as subjects eight normal human males.

Total-body iron loss amounted to 1.5 mg per day, or 0.05%, if total-body radioiron loss rate represents total-body iron loss.

The radioiron loss curve after the death of a labeled red cell generation was found on the average to be nearly parallel to the initial loss curve.

Reutilization of radioiron occurs after the death of labeled red cells, and this leads to change of distribution of radioiron in the body. Thus it is suggested that a decrease of whole-body count after 100 days can be better explained by changes of geometry and greater absorption of gamma rays, due to the change of radioiron distribution in the body, than by a small amount of radioiron loss.

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Normal Response to Erythropoietin or Hypoxia In Rats Made Anemic with Turpentine Abscess

Abraham Gutnisky and Donald Van Dyke

It is apparent that the anemia which develops in the presence of inflammation has a complex etiology. Disturbances in iron metabolism result in a markedly lowered plasma-iron concentration and shunting of iron to storage pools (1,2,3,4). Wintrobe (5) has shown that the anemia of inflammation will disappear with the administration of cobalt, which is believed to act through stimulation of erythropoietin production (6). Yet in a preliminary study, Erslev (7) obtained evidence suggesting that both the production and the effectiveness of erythropoietin were defective in the presence of inflammation. The present study was designed to clarify the role of erythropoietin in the etiology of the anemia that follows the production of a sterile turpentine abscess by testing the ability to respond to both exogenous erythropoietin and endogenous erythropoietin (hypoxia).

MATERIALS AND METHODS

Specific pathogen-free, adult, female rats of the Sprague-Dawley strain, weighing approximately 200 g and fed ad libitum on Diet #1,* were used throughout the study except for rats of the Long-Evans strain used to make the erythropoietin dose-response curve (8). Turpentine abscesses were produced by injection of N. F. oil of turpentine into the gluteal muscle (0.125 ml on days 1 and 2 and 0.250 ml on day 7). On day 15, the rats were weighed, and the total circulating red-cell volume and hemoglobin were determined by the Fe^{59}-labeled-red-cell-dilution method (9). Leukocytes and reticulocytes were counted. Bone-marrow smears were made from femoral marrow diluted with rat serum. The weight of the spleen was recorded. Determination of the plasma-iron concentration and total iron-binding capacity of the plasma was determined on plasma pooled from rats of the same group (10).

Erythropoietin was obtained by the collodion adsorption method (11) from the urine of a patient who had complete red-cell aplasia. It had been assayed by a variety of methods (8). One-half or 2.5 Comparative Standard (C.S.) (12) unit was given daily subcutaneously for 14 days, starting the day of the first

*The diet, obtained from Simonsen Laboratories, Gilroy, Calif., consisted of 59.0% wheat, 11.7% skim milk, 11.2% casein, 11.2% rice bran, 3.3% vegetable oil, 1.3% $CaCO_3$, 0.7% NaCl and vitamin and mineral mixtures to make up 100%.

Table 1. Effect of a small dose of erythropoietin on hematologic values, body and spleen weight of normal and turpentine-treated rats* (0.5 C.S. units per day for 14 days)

Measurement	Normal untreated	Turpentine only	Normal + ESF	Turpentine + ESF
Total circulating red-cell volume, ml	5.48 ± .33†	4.51 ± .24	6.62 ± .48	5.02 ± .36
Hematocrit, %	41.4 ± 1.66	38.0 ± 2.01	49.1 ± 2.17	42.7 ± 2.37
Hemoglobin, g/100 ml	12.5 ± .59	11.3 ± .57	14.2 ± .83	12.4 ± .70
Reticulocytes, %	1.8 ± .52	7.9 ± 2.34	4.8 ± .42	6.6 ± 2.04
Spleen weight, mg	570 ± 91	852 ± 102	595 ± 104	903 ± 225
Body weight, initial, g	194 ± 9.6	194 ± 8.9	193 ± 11.4	194 ± 9.2
Body weight, final, g	238 ± 11.7	221 ± 10.1	234 ± 10.4	217 ± 14.0

*6 female rats of the Sprague-Dawley strain in each group.

†Standard deviation.

turpentine injection. The controls were given saline. Exposure to hypoxia was accomplished by placing rats, with or without a turpentine abscess, in a decompression chamber at a simulated altitude of 20,000 ft for 14 days, starting the day of the first injection of turpentine.

RESULTS

As shown in the first two columns of Tables 1, 2 and 3, turpentine abscess caused a mild anemia, with reduction in total circulating red-cell volume of 18% ($P<0.001$*), in total circulating hemoglobin of 15% ($P<0.001$) compared to the untreated controls, a significant increase in reticulocytes ($P<0.001$), and a suggestion of a reduction in percent erythroblasts in the marrow ($P<0.02$). There was a steady rise in the percent reticulocytes in the controls, which is assumed to be the result of stimulation of erythropoiesis due to blood loss from daily sampling. Following administration of turpentine, there was no increase in reticulocytes during the first few days, but after the 6th day the percent reticulocytes increased to twice the number in the controls (Fig. 1). Both large and small doses of erythropoietin increased the hemoglobin, hematocrit, total circulating red-cell volume, and total circulating hemoglobin (Table 1 and 2).

*Fisher "t" test.

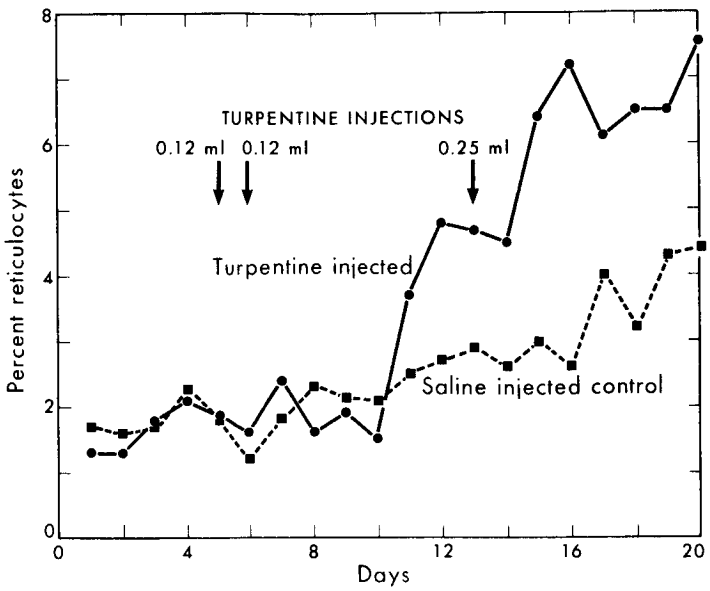

Figure 1. Percent reticulocytes in peripheral blood in normal rats and in rats following production of a turpentine abscess. The gradual increase in reticulocyte count in the normal controls is assumed to be the result of blood loss through daily sampling.

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Table 2. Effect of large dose of erythropoietin on hematologic values, body and spleen weight of normal and turpentine-treated rats (2.5 C.S. units per day for 14 days)

Measurement	Normal untreated (6)*	Turpentine only (5)	Normal + ESF (6)	Turpentine + ESF (4)
Total circulating red-cell volume, ml	5.44 ± .54†	4.21 ± .63	9.41 ± 1.17	7.71 ± 1.73
Hematocrit, %	41.5 ± 2.15	36.1 ± 3.16	55.4 ± 3.16	51.0 ± 5.65
Hemoglobin, g/100 ml	13.4 ± .60	12.6 ± .38	17.1 ± .93	16.0 ± .83
Plasma iron, µg/100 ml	358	262	72	127
Total iron binding capacity, µg/100 ml	785	704	584	657
Reticulocytes, %	2.8 ± .93	6.9 ± 2.08	7.9 ± 1.08	9.7 ± 2.44
Marrow erythroblasts, %	18.3 ± 9.5	13.1 ± 8.3	28.8 ± 8.3	23.2 ± 3.4
Leucocytes/mm^3 x 10^3	12.600 ± 4.12	12.080 ± 3.31	12.057 ± 2.11	13.962 ± 2.52
Spleen weight, mg	666 ± 276	716 ± 232	1,064 ± 238	1,182 ± 150
Body weight, initial, g	204 ± 6.63	204 ± 14.6	204 ± 10.7	200 ± 13.4
Body weight, final, g	242 ± 14.7	225 ± 27.8	247 ± 11.0	225 ± 17.1

*Number of female rats of the Sprague-Dawley strain in each group is indicated in parentheses.

†Standard deviation.

Table 3. Effect of hypoxia on hematologic values and body weight of normal and turpentine-treated rats (20,000 feet for 14 days)

Measurement	Normal untreated (6)*	Turpentine only (3)	Normal + hypoxia (5)	Turpentine + hypoxia (6)
Total circulating red-cell volume, ml	5.31 ± .37†	4.61 ± .45	8.10 ± .63	7.64 ± 1.11
Hematocrit, %	42.7 ± 1.66	38.3 ± 1.83	63.0 ± 3.16	56.8 ± 2.46
Hemoglobin, g/100 ml	13.4 ± .55	12.2 ± .50	18.7 ± .83	16.7 ± 1.60
Reticulocytes, %	1.5 ± .50	2.8 ± 1.57	4.4 ± 1.72	4.7 ± 2.50
Marrow erythroblasts, %	20.7 ± 10.9	8.6 ± 2.97	29.7 ± 11.8	22.9 ± 4.8
Leucocytes/mm^3 x 10^3	12.191 ± 1.88	11.141 ± 1.11	10.450 ± 1.93	12.050 ± 1.33
Body weight, initial, g	197 ± 13.2	198 ± 7.7	197 ± 13.3	201 ± 14.5
Body weight, final, g	243 ± 26.5	215 ± 15.9	202 ± 11.1	200 ± 17.5

*Number of female rats of the Sprague-Dawley strain is indicated in parentheses.

†Standard deviation.

At the lower dose of erythropoietin this resulted in repair of the anemia produced by turpentine, and at the higher dose in the production of polycythemia in the presence of a turpentine abscess. For each dose, the percentage increases in total circulating red-cell volume were essentially identical in rats with and without abscesses.

Both the normal and turpentine-treated rats showed a marked increase in hematocrit, hemoglobin, total circulating red-cell volume and total circulating hemoglobin in response to hypoxia (Table 3). The increase in total circulating red-cell volume was 70% in the rats having a turpentine abscess and 68% in the rats having no abscess. The total circulating hemoglobin increased in proportion to the total circulating red-cell volume.

DISCUSSION

The production of a turpentine abscess was followed by the development of a mild anemia characterized by a reduction in total circulating red-cell volume and total circulating hemoglobin of approximately 16%. This degree of anemia is comparable to that reported by previous workers (Wintrobe *et al.* (5)).

The administration of either large or small doses of human urinary erythropoietin produced the same percent increase in hemoglobin, hematocrit and total circulating red-cell volume in rats having a turpentine abscess as in normal rats, although the final values fell short of those obtained in the normal rats by the same difference as that in the respective controls. Thus, although the rats with an abscess showed no deficiency in response to erythropoietin, the administration of erythropoietin did not negate the defect produced by the turpentine abscess. Since there is a log-dose response relationship to erythropoietin (8), a large dose such as was used in this experiment would be expected to negate any small differences in endogenous erythropoietin titer which might have existed in the rats if the etiology of the anemia were based on inadequate endogenous erythropoietin levels. The fact that the defect persisted after large doses of erythropoietin were given indicates that it is based on some factor other than an inadequate level of erythropoietin in the circulation.

Exposure to hypoxia (a simulated altitude of 20,000 ft for 14 days) resulted in the same percent increase in hemoglobin, hematocrit, and total circulating red-cell volume in the rats having a turpentine abscess as in the normal controls, although the values in rats with an abscess again fell short of those obtained in the normal rats by the same difference as that in the respective controls. These results indicate that the rat having a turpentine abscess is capable of producing and utilizing erythropoietin normally when stimulated. This suggests that the anemia which accompanies the presence of a turpentine abscess is not the direct result of a defect in erythropoietin metabolism, but that the mild anemia does not serve as an adequate stimulus to increased erythropoietin production. A turpentine abscess was chosen as an easily standardized method of producing an inflammatory lesion with the realization that inflammatory lesions from other agents such as bacterial (13) or antigenic (14) may give quite different results.

SUMMARY

Rats with a turpentine abscess developed characteristic mild anemia. Such rats, after treatment with human urinary erythropoietin or exposure to hypoxia, showed an erythropoietic response comparable to that of the normal controls. Therefore, the marrow is capable of responding when stimulated, suggesting that the mild anemia which accompanies a turpentine abscess does not serve as an adequate stimulus for increased red-cell production.

ACKNOWLEDGEMENTS

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Preliminary Results from the Use of Erythropoietin In Human Volunteers

Donald Van Dyke, John H. Lawrence, Myron Pollycove and Peter Lowy

The therapeutic usefulness of erythropoietin is made doubtful by the fact that one frequently finds high titers of erythropoietin in severely anemic individuals. However, in many anemic persons no elevation of titer can be demonstrated with present assay methods, and the possibility exists that among these there may be some in whom a deficiency in erythropoietin production is a primary etiologic factor. In order to test this possibility by therapeutic trial, it was first necessary to determine what preparation, at what dose, and for what duration would constitute adequate therapeutic trial in man. It was felt that the most meaningful starting point would be to ascertain the minimum effective dose of human erythropoietin in normal human beings. With this object in mind, the effectiveness of human urinary erythropoietin was studied in normal human volunteers.

During the investigation of the dose of human urinary erythropoietin necessary to stimulate erythropoiesis in normal human beings, the volunteers who received the active urine or urinary extract developed high platelet counts concomitant with evidence of increased red-cell production. The erythropoietin used was obtained from the urine of a patient with red-cell aplasia and thrombocytopenia. Subsequent investigation in rats showed that the fraction used, which was erythropoietically active in rats, also produced an increase in platelet count proportional to the amount of material injected.

MATERIALS AND METHODS

The urinary erythropoietin concentrate and the unfractionated urine used in the human volunteers and in the studies on rats were obtained from a male patient with complete red-cell aplasia who subsequently developed thrombocytopenia (1). The concentrate was prepared by adsorption on collodion, precipitation in ether alcohol, and differential solubility as previously described (2). This preparation had proved to be pyrogenic in earlier human trials. Therefore, one further step, ethanol fractionation, was added and the product proved to be non-pyrogenic. A 2% w/v solution of erythropoietin in saline containing 0.1% phenol was boiled for 30 sec, and then chilled in ice. 1.3 vol of 100% ethanol were added. The next day the precipitate was removed by centrifugation and 2.7 vol (referred to the volume of the original aqueous solution) of

ethanol were added to the supernatant. The resulting precipitate was collected after 3 hr, washed with ethanol and ether, and dried *in vacuo*. All operations were done at 5° C.

Dose-response curves for the erythropoietin concentrate were obtained by the Fe^{59} red-cell-incorporation assay (3) in starved rats and in hypophysectomized rats, by the total-circulating-red-cell-volume assay (4) in normal and hypophysectomized rats, and by the ability of erythropoietin to stimulate Fe^{59} incorporation and reticulocytosis in hypertransfused mice (5). Results using hypophysectomized rats will be reported in detail elsewhere.

Blood-volume determinations in the human volunteers were done by the *in vitro* Cr^{51} labeled erythrocyte method (6). Reticulocyte counts were done by the dry brilliant cresyl blue method and counterstained with Giemsa. One thousand cells were counted on each slide. Platelet counts were done using Unopettes.*

Rats of the Long-Evans strain fed *ad lib*† were used throughout these studies.

Erythropoietin concentrated from the urine by the collodion adsorption method (2) alone had previously been pyrogenic in five human volunteers, necessitating termination of the study after one or two injections. It was assumed that the pyrogenicity was a product of the extraction method, and since the unmodified urine contained a high titer of erythropoietin, it was decided to infuse the active urine without fractionation. The urine was frozen immediately after donation in sterile, pyrogen-free bottles. It was infused intravenously without further handling after a millipore bacterial filter (0.22 microns) was placed in the line. The urine was infused over a six-hour period, and the bottle and the millipore filter were kept cool with ice. Samples of the urine in the bottle and samples taken after passage through the filter were collected and assayed by the Fe^{59} red-cell-incorporation assay in starved rats.

One recipient was a 47-year-old woman weighing approximately 60 kg. Because both patients were in casts, no accurate weight was possible, and for this reason the results are expressed as total circulating red-cell volume (RCV) rather than RCV/kg body weight. This woman had been in the hospital for two and a half months, immobilized in traction for a fractured femur and pelvis. She had had no recent transfusions, operations, manipulations, or infections. She had no known hematologic abnormality, and her hemogram was within normal limits

*Unopette; Becton, Dickinson and Company.

†The diet was obtained from Simonsen Laboratories, Gilroy, California. It consisted of 59.0% wheat, 11.7% skim milk, 11.2% casein, 11.2% rice bran, 3.3% vegetable oil, 1.3% $CaCO_3$, 0.7% NaCl, and vitamin and mineral mixtures to make up 100%.

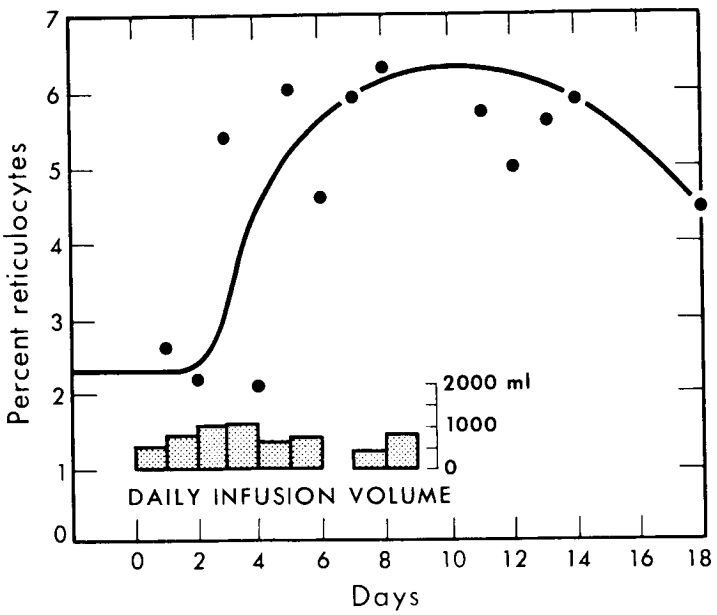

Figure 1. Increase in reticulocyte count in an erythropoietically normal human volunteer during and following the intravenous administration of erythropoietically active urine. (Total dose: 480 C.S. units)

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at the onset of the study. The subject received an average of 828 ml of urine per day for a period of 8 days.

The other recipient of the urinary concentrate was a 50-year-old man, weighing approximately 60 kg, in traction for multiple fractures. He had been in the hospital 3 months and had had no recent blood transfusions, operations, or manipulations, but had an infected non-union of the lower third of the tibia. He had no known hematologic abnormality, and his hemogram was normal at the onset of the study. This subject received a dose of 100 mg of the final alcohol precipitate dissolved in saline in a single subcutaneous injection daily for 7 days. The erythropoietic activity of various batches of the material differed so that the patient received 425, 358, 358, 358, 147, 147, and 147 C.S. (Comparative Standard) (7) units per day respectively.

RESULTS

An average of 828 ml of erythropoietically active human urine, unmodified except for being passed through a millipore bacterial filter, was infused into a normal human volunteer daily for 8 days. The volume infused each day is indicated in Fig. 1. The infusion was given over a six-hour period. A mild fever accompanied by a slight chill and nausea developed toward the end of the infusion on the 3rd, 4th, 6th, and 8th days. These were the only signs or symptoms of reaction and they disappeared quickly following termination of the infusion.

A composite sample of the urine that the patient received (after the

Table 1. Loss of erythropoietin in passage through millipore filter (10 starved rats in each group)

Treatment of urine	Total dose ml	Fe^{59} uptake %
None	8	15.5 ± 0.8*
Sample after 100 ml passed filter	8	4.8 ± 0.6
After 700 ml	8	14.9 ± 1.3
Material recovered from the filter† after 2000 ml had passed	2	16.4 ± 1.4
Uninjected control	-	4.7 ± 0.2

*Standard error of the mean.

†Millipore dissolved in acetone. Residue suspended in 24 ml saline.

millipore filter), collected throughout the period of infusion, gave an erythropoietic response in starved rats equivalent to 0.07 C.S. units per ml. This was approximately 25% of the activity of the urine collected from the system just before passage through the millipore filter. When a small volume (150 ml) of active urine was passed through a millipore filter, no activity was recovered in the filtrate. Very little activity was recovered from the filter membrane itself, suggesting that loss is primarily through inactivation rather than adsorption. The results of one experiment illustrating the loss of erythropoietic activity in passing through the millipore filter are summarized in Table 1. The fact that erythropoietic activity appeared in the filtrate after a considerable volume of urine had passed suggests that the filter becomes altered (saturated) in some way. When aliquots of the same urine were passed through sintered glass or Seitz bacterial filters, no activity was recovered in the filtrate even after large volumes (2,000 ml) had been passed.

The reticulocyte response in the normal human volunteer given infusions of active urine modified by the millipore filter is summarized in Fig. 1. As can be seen, there was an increase in reticulocyte count within a few days after starting the infusion, and this increase was maintained for a period of at least 10 days. There was no significant change in hemoglobin, hematocrit, leucocyte count, or total circulating red-cell volume during the 18-day period the patient was followed. On the 5th day of treatment it was observed that the blood smears contained what appeared to be an excess of platelets in large clumps. A platelet count done at that time gave a value of 660,000. A platelet count done 9 days after cessation of treatment was in the low normal range (248,000).

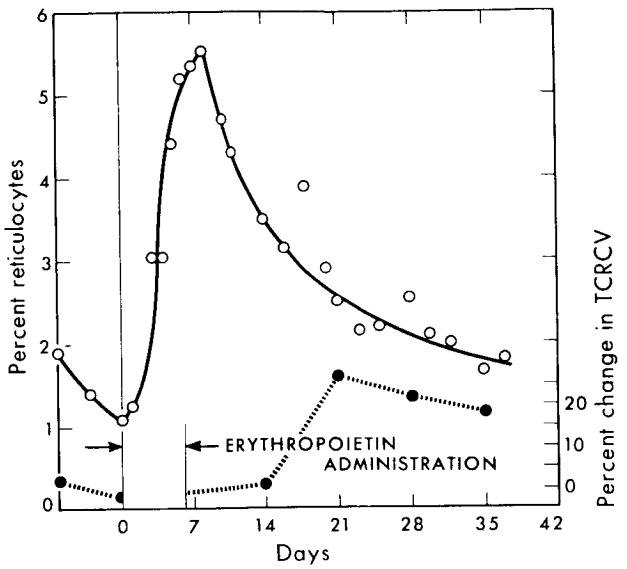

Figure 2. Increase in reticulocyte count (above) and total circulating red-cell volume (below) in an erythropoietically normal human volunteer following treatment with human urinary erythropoietin. (Total dose: 1940 C.S. units)

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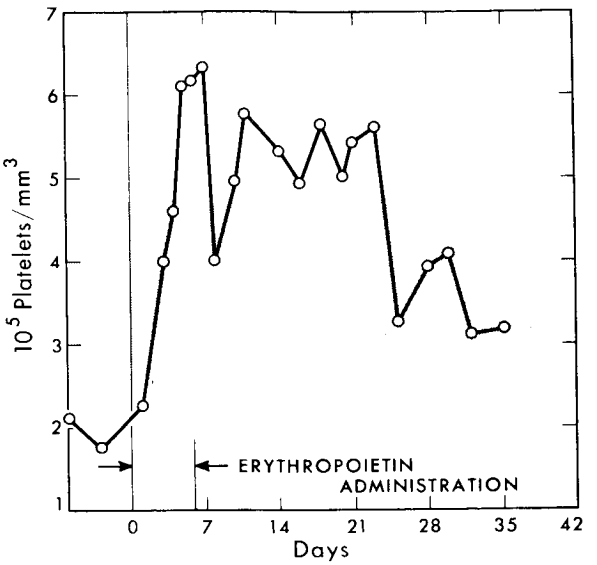

Figure 3. Increase in platelet count in an erythropoietically normal human volunteer following treatment with erythropoietin obtained from the urine of an anemic, thrombocytopenic donor. The shaded area indicates the normal range in this laboratory.

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Because of the high platelet count observed during the above study, the platelet count of the next human volunteer was followed before, during, and after treatment. The second subject was given concentrated human urinary erythropoietin as a single subcutaneous injection daily for 7 days. After this time the treatment was stopped for fear the platelet count, which had increased to over 600,000, would rise to levels where spontaneous thrombosis would occur. The patient developed redness, swelling, and tenderness at the injection sites but developed no fever, nausea, malaise, or other signs or symptoms of systemic reaction.

The reticulocyte count rose abruptly during treatment and began to fall 2 days after cessation of treatment, Fig. 2. As can be seen, the total circulating red-cell volume was found to be increased by 28% on the 21st day of the study. The platelet count rose abruptly during the period of treatment and remained above normal values for a period of 17 days following treatment, Fig. 3. The leucocyte count rose abruptly during the period of treatment and promptly fell to normal levels when treatment was stopped, Fig. 4. Differential counts showed that the rise in leucocyte count was due to increased numbers of both granulocytes and lymphocytes.

On the basis of the apparent increase in numbers of platelets in the peripheral blood of the two human volunteers, it was decided to investigate the

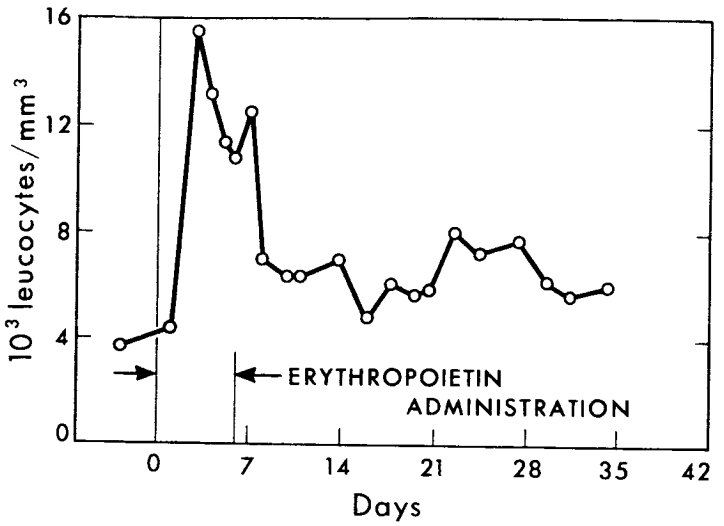

Figure 4. Increase in leucocyte count of an erythropoietically normal human volunteer during treatment with human urinary erythropoietin. Both granulocytes and lymphocytes were increased.
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platelet stimulating properties of the urine and the urinary extract from the same donor, using rats as recipients.

Platelet counts were done on normal adult female rats every 3 days for 2 weeks to establish the normal variations. The rats were then given 2 ml of active urine subcutaneously daily for 6 days. By the 5th day of treatment, the platelet count had nearly doubled and remained elevated for 5 days. On the basis of this experiment, the following assay schedule for thrombopoietic activity was chosen: Platelet and reticulocyte count on day 1; no treatment on days 2 and 3; a single subcutaneous injection of test material on days 4 and 5; repeat platelet and reticulocyte count on day 8. With 2 ml of active urine, an increased platelet count was consistently obtained, and no response was obtained with urine from 2 normal subjects.

Since both unmodified urine and urinary extract appeared to increase the platelet count in human volunteers, various doses of the concentrate were tested in rats, using the same time schedule in the assay as above. The results are summarized in Table 2 and Fig. 5. From Table 2 it can be seen that the response for both platelets and reticulocytes was proportional to the dose of erythropoietin given. Fig. 5 is a plot of the percent increase in number of platelets as a function of the log of the dose of erythropoietin. The limited data are compatible with a log dose response relationship.

DISCUSSION

In a previous study (2) it was estimated that the dose required to produce a 50% increase in total circulating red-cell volume in either rats or monkeys is approximately 6 C.S. units (or 60 cobalt units) per kg of body weight per day for 14 days. On this same basis, a 60 kg man (the approximate weight of both subjects in this study) would require 360 C.S. units per day to produce

Table 2. Effect of various doses of human urinary erythropoietin on the platelet and reticulocyte count of normal rats

Total dose mg	Platelets before	Platelets after	Reticulocytes before	Reticulocytes after
	10^6 cells/mm^3		%	
	0.76	1.12		
	1.17	1.92		
	0.96	1.70		
20	0.83	1.27	3.2	12.5
	1.03	1.59	4.6	18.2
	0.83	1.27	1.8	14.5
	0.89	1.37	2.0	13.6
	1.13	1.59	3.9	18.7
Average	0.95	1.48	3.1	15.5
	0.97	1.03		
	0.75	1.76		
	1.14	1.18		
2	0.96	1.51		
	0.78	1.30	3.5	10.1
	0.96	0.91	2.0	13.4
	1.04	1.31	4.9	11.6
	0.82	1.50	3.5	10.5
	1.06	1.23	2.8	10.4
Average	0.94	1.30	3.3	11.2
	0.80	0.97		
	1.01	1.32		
0.2	1.12	1.10		
	0.90	1.16		
	1.15	1.02	3.7	5.7
	0.84	1.00	4.6	7.9
	0.88	1.09	5.8	8.3
	0.81	0.96	2.5	5.3
	0.99	1.21	2.5	4.7
Average	0.94	1.09	3.8	6.4

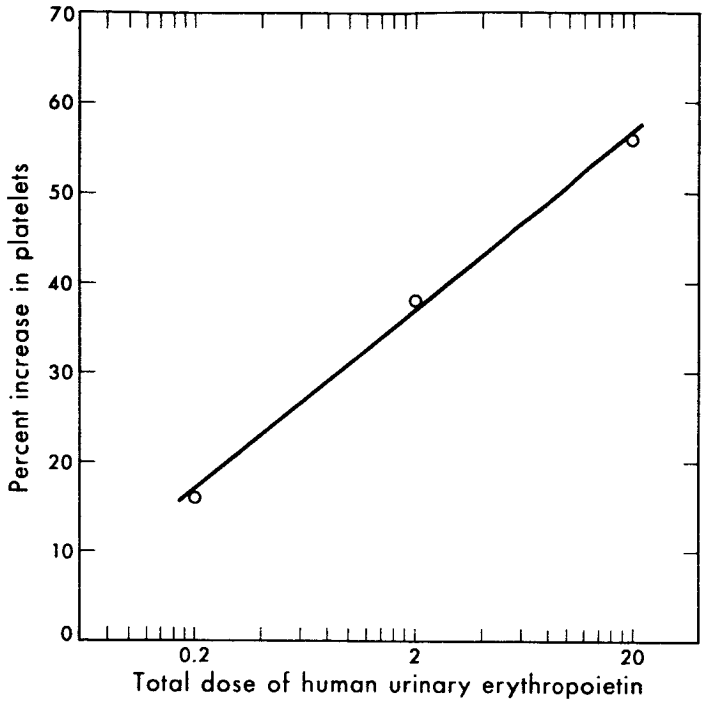

Figure 5. Percent increase in platelet count obtained in normal rats with various doses of erythropoietin obtained from the urine of an anemic, thrombocytopenic donor.
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a 50% increase in red-cell volume. Since a 25% increase requires one-third that dose in rats, it was predicted that one might produce a significant increase in a normal human subject with 120 C.S. units per day for 14 days.

Because of the loss of erythropoietin incurred on passage of the urine through a millipore filter, the first human volunteer received only one-fourth the predicted dose or approximately 60 C.S. units per day, or a total dose of 480 C.S. units over the 8-day period. Thus the dose received by the first human recipient would be expected to produce a reticulocytosis but not a significant increase in circulating red-cell volume. Such a result was obtained. Reticulocytosis alone is not considered conclusive evidence for increased erythropoiesis.

The second human volunteer received an average dose of 277 C.S. units per day for 7 days. Judging from previous work on monkeys and rats, such a dose if given over a 14-day period would be expected to give an increase in total circulating red-cell volume of between 25 and 50%. With only 7 days of treatment, the red-cell volume of the patient increased by 27%. The timing of the reticulocyte response and the red-cell-volume increase are such that it is concluded that erythropoiesis was stimulated and that the requirements in man (dose/kg) are in the same order of magnitude as in monkey and rat.

These limited data suggest that in normal humans one could expect a significant reticulocyte response from the injection of 2 C.S. units (20 cobalt units) per kg per day for 4 days. To obtain a significant increase in total

circulating red-cell volume would probably require a dose of 5 C.S. units (50 cobalt units) per kg daily for a period of 7 to 14 days.

In both the human volunteers studied, a high platelet count was found during the treatment and in the second subject, where pre- and post-treatment counts were made, it is apparent that the elevated platelet count corresponded with and was, therefore, probably the result of the administration of the human urinary extract. Administration of the same material to rats consistently resulted in a small but definite increase in platelet numbers, the increase being proportional to the logarithm of the dose. It seems that the urine contained some factor capable of increasing the number of platelets in the circulation of both man and rats, and that this factor appeared in the same fraction as erythropoietin after collodion adsorption, ether-alcohol precipitation, differential solubility, lyophilization, and alcohol fractionation. Possibly the presence of this platelet increasing factor in the urine is related to the thrombocytopenia of the patient and is analagous to the relationship between urinary erythropoietin and anemia.

Many observations in the last few years have indicated the possibility that thrombopoiesis is controlled by "humoral" mechanisms similar to those controlling erythropoiesis (8-12). Since the patient from whom the urine was obtained had both severe anemia and thrombocytopenia, it is a temptation to assume that the fraction extracted from his urine contained two specific factors, erythropoietin and thrombopoietin. However, the present study provides no basis for such an assumption as it has not been possible as yet to compare the extract used in these studies with similarly prepared extracts from: anemic patients with normal platelet counts, non-anemic thrombocytopenic patients, or extracts from the urine of normal subjects. Similarly, one is tempted to assume that the increased platelet numbers result from an increased rate of production of platelets, but other possibilities have not been ruled out.

Linman (10) has suggested that a single factor may affect the proliferation of all blood-cell precursors and may explain the leucocytosis and thrombocytosis in certain types of hemolytic anemia, after acute hemorrhage, and in patients with polycythemia vera. In the second human subject of this study, increases in erythrocytes, leucocytes, and platelets were clearly associated with the treatment. The leucocytosis was the result of an increase in granulocytes and lymphocytes and was presumably due to the administration of a crude mixture of denatured proteins rather than to the presence of "leucopoietin." Leucocytosis did not occur in the patient who received unmodified urine. The erythropoietin preparation used has consistently failed to influence the leucocyte count in rats; however, problems of species specificity may justify careful evaluation of all blood-cellular elements in future trials of erythropoietin in human beings.

SUMMARY

An attempt is made to determine the minimum effective dose of erythropoietin in man from study of two erythropoietically normal human volunteers. One received an intravenous infusion of erythropoietically active urine from an anemic, thrombocytopenic donor. The other was given an extract of urine from the same donor. The results indicate that the minimum dose necessary to produce reticulocytosis in a normal human being is approximately 2 C.S. units/kg/day for a period of several days. The dose necessary to produce an unquestionable increase in total circulating red-cell volume is estimated to be approximately 5 C.S. units/kg/day for 7-14 days.

Higher than normal platelet counts were associated with treatment in both subjects studied, suggesting that the donor excretes in his urine both erythropoietin and thrombopoietin.

ACKNOWLEDGEMENTS

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Immunological Neutralization of Various Erythropoietins

Joseph F. Garcia and John C. Schooley

Serum from rabbits immunized with an extract of erythropoietically active human urine neutralizes in vitro the erythropoietic activity of such extracts. In addition, injections of such immune serum into normal mice depress erythropoiesis, indicating that endogenous erythropoietin of mice can be neutralized and that normal erythropoiesis in mice is erythropoietin dependent. Both neutralizations are considered to be the result of an antigen-antibody reaction directed against either erythropoietin or the general class of molecules to which erythropoietin belongs (1,2).

Erythropoietically active material has been found in plasma and in urine of several animal species. Some of these erythropoietins have been effective in stimulating erythropoiesis when injected into animals which differ in species from the source of the administered erythropoietin (3). The purpose of the present investigation is to study the inter-species relationships of erythropoietin using immunological methods.

MATERIALS AND METHODS

A urine extract from a patient with aplastic anemia was prepared by ultrafiltration through a collodion membrane. This extract had erythropoietic activity equivalent to approximately 20 cobalt units per mg dry weight. An alum precipitate of the urinary extract was prepared by the method of Kabat and Mayer (4). Rabbits were immunized with a suspension of the alum-precipitated urinary erythropoietin containing 1.5 mg of extract per ml. The rabbits were immunized by intravenous injection three times a week, the first injection being given intraperitoneally. The volume of the alum-precipitated suspension injected varied as follows: three injections of 1 ml; three injections of 1.5 ml; four injections of 2 ml; four injections of 3 ml, and two injections of 5 ml. The animals were bled on the 5th to 7th day after the final injection, and the serum was collected. The ability of such serum to neutralize erythropoietin was determined by its effect in depressing the 24-hour red-blood-cell incorporation of Fe^{59} in normal mice after 4 daily subcutaneous injections of 0.25 ml of serum per day. The immune serum used in this study depressed the Fe^{59} incorporation to 1.5% from a value of 26.9% for mice similarly treated with normal rabbit serum.

A hypoxic environment or production of a severe anemia by injection of phenylhydrazine was used to elevate the serum erythropoietin levels. Groups of rats and rabbits and a single sheep were exposed in a decompression chamber to an oxygen tension equivalent to that of 20,000-ft altitude (approximate pO_2 = 72 mm Hg). All animals were bled within 30 minutes after 24 hours of hypoxic exposure; the sheep was bled again after 48 hours of hypoxic exposure. Phenylhydrazine anemia was produced in other groups of rabbits and mice. The rabbits were injected daily for 6 days with a solution of 2.5% phenylhydrazine hydrochloride, 2 ml on the first day and 1 ml on each subsequent day, and then bled the day following the last injection. Mice were given 4 injections of 1.5 mg phenylhydrazine hydrochloride per injection over a 6-day period and bled on the 7th day.

Mice with transfusion-induced polycythemia were used for the erythropoietin assay in a procedure slightly modified from DeGowin et al. (5). Groups of 10 female Swiss mice, each animal weighing 20-25 g, were given 1 ml of washed red blood cells intraperitoneally on 2 successive days. On the 5th day following the last transfusion the test material was given subcutaneously. Two days later approximately 0.5 μC of Fe^{59} (approximately 0.05 μg of iron) was given intraperitoneally. The mice were bled 3 days after injection of the radioiron and the radioactivity of 0.5 ml of blood was determined. Values from any animal losing weight or whose hematocrit was less than 55% at the time of autopsy were discarded. Results are expressed as the percent of the injected dose of Fe^{59} in the calculated total blood volume. Blood volume of these hypertransfused mice was assumed to be 7.0% of the total body weight.

Neutralization of erythropoietin in the various animal sera was determined by comparing the erythropoietin activity after mixing and incubating 9 ml of each serum with 2 ml of the serum obtained from the rabbits immunized with erythropoietin (hereafter referred to as anti-E) or with 2 ml of normal rabbit serum. Treated sera were incubated at 37°C for 1 hr and then placed in a refrigerator (10°C) overnight. After centrifugation, 1 ml of the supernatant was injected subcutaneously into each assay mouse. The ability of the anti-E to neutralize an extract of erythropoietin obtained from phenylhydrazine anemic sheep plasma* was also determined. This extract was dissolved in 9 ml of saline and the neutralization was performed in the same manner as for the various sera. Similar neutralizations were carried out using serum and urinary extract from the patient whose erythropoietin was used for immunizing the rabbits. The various sera were kept frozen until ready for use.

RESULTS

Increased amounts of erythropoietin were found in sera taken from the

*Al-0336, No. K103194A obtained from Hematology Study Section, National Institutes of Health.

rats, rabbits, and from the one sheep exposed to a simulated 20,000-ft altitude. The amount of erythropoietin found in serum of the three species after the same duration of exposure to altitude was noticeably different. The largest amount of erythropoietin was found in serum of the rat and the smallest in serum of the sheep. The data presented in Table 1 indicate that erythropoietic activity of the sera of these animals was completely neutralized by anti-E.

Table 1. Neutralizing effect of immune serum on erythropoietic activity of sera from various species

Source of serum		Percent radioiron incorporation in the calculated total red cell volume	
		Normal rabbit serum	Anti-E serum
Rat:	Altitude (24 hr at 20,000 ft)	37.46 ± 1.85*	0.28† ± 0.03
Sheep:	Altitude (24 hr at 20,000 ft)	2.67 ± 0.63	0.17‡ ± 0.02
	Altitude (48 hr at 20,000 ft)	3.78 ± 0.99	0.20‡ ± 0.05
Rabbit:	Altitude (24 hr at 20,000 ft)	11.36 ± 0.89	0.38† ± 0.07
	Phenylhydrazine-treated	13.56 ± 1.41	1.41† ± 0.34
Mouse:	Phenylhydrazine-treated	25.90 ± 2.66	0.16† ± 0.01
Human:	Aplastic anemia	15.05 ± 1.50	0.52† ± 0.11
Saline Control:		0.18 ± 0.04	

*Standard error of the mean

†P value <0.001. Significance between respective sera treated with normal rabbit serum and anti-E.

‡P value <0.01. Significance between respective sera treated with normal rabbit serum and anti-E.

Increased amounts of erythropoietin were found in sera of the mice and of the rabbits injected with phenylhydrazine. The data presented in Table 1 also indicate that the erythropoietic activity of sera of phenylhydrazine-treated mice was completely neutralized by incubation with anti-E. Similar treatment of sera obtained from phenylhydrazine-injected rabbits caused a significant neutralization of the erythropoietic activity of such sera. However, in this case, some erythropoietin remained unneutralized, since radioiron incorporation into red blood cells was significantly higher than for a group of

saline-injected controls, with a P value of 0.001 (according to Fisher's table of "t").

Erythropoietic activity of serum obtained from the aplastic anemic patient, whose urine was the source of erythropoietin used for immunizing the rabbits, was completely neutralized by incubation with anti-E (Table 1).

Neutralization by anti-E is shown in Table 2 for erythropoietic activity of extracts made from urine of an aplastic anemic patient, and from plasma of sheep made anemic by injections of phenylhydrazine. Erythropoietic activity of the urinary extract was completely neutralized. This confirms our previous findings for erythropoietic extracts from the urine obtained from a different patient (1). Erythropoietic activity of sheep plasma erythropoietin was significantly, but not completely, neutralized by anti-E. Unneutralized erythropoietin caused a radioiron incorporation which was significantly higher than that observed in the saline-injected controls ($P < 0.001$).

Table 2. Neutralizing effect of immune serum on erythropoietin extracts

Extracts	Percent radioiron incorporation in the calculated total red-cell volume	
	Normal rabbit serum	Anti-E serum
Phenylhydrazine anemic sheep plasma	13.51 ± 1.36*	3.91† ± 0.50
Human urinary erythropoietin	11.88 ± 1.11	0.28† ± 0.07
Saline control	0.18 ± 0.04	

Approximately 1 cobalt unit of each extract was given to each assay animal.

*Standard error of the mean.

†P value <0.001. Significance between extracts treated with normal rabbit serum and anti-E.

DISCUSSION

The present investigation demonstrates that the biological activity of erythropoietins found in serum of mice, rats, rabbits, sheep, and humans can be neutralized by the addition of serum obtained from rabbits immunized with human urinary erythropoietin. Significant neutralization of erythropoietic activity was observed in all cases. In all but two instances it was complete. In these

two cases phenylhydrazine anemia was used as a stimulus for increased erythropoietin levels. While this may be of importance, other factors may be involved such as small species differences in the erythropoietin molecule. The results suggest that erythropoietins from the various animal sources investigated here are similar.

Rabbit anti-E was found to neutralize rabbit serum erythropoietin in vitro. This raises an interesting question. Did neutralization of endogenous erythropoietin take place in the rabbits being immunized? It was noted at the time of harvesting the antiserum that most of the rabbits having high anti-E titers had hematocrits well below normal. This suggests that some neutralization of endogenous erythropoietin in the rabbits being immunized did, in fact, occur, i.e., an autoimmune condition results. A more extensive analysis of erythropoiesis in the rabbits being immunized is in progress.

SUMMARY

Increased serum erythropoietin levels were produced in mice, rats, rabbits, and in a sheep in response to hypoxia or phenylhydrazine anemia. In all cases, including that of the rabbit, the serum erythropoietin was neutralized on addition of serum from rabbits previously immunized with an erythropoietin extract of human urinary origin. In addition, such serum neutralized an erythropoietin extract perpared from plasma taken from a phenylhydrazine-anemic sheep. These data suggest that erythropoietins of the various animal species studied are similar.

ACKNOWLEDGEMENTS

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Correlation Between Erythropoietin Titer, Evidence of Tissue Hypoxia and Hemoglobin Synthesis In a Normal Human Volunteer Acutely Exposed to Hypoxia

William Siri, Donald Van Dyke, H. Saul Winchell, Myron Pollycove and Howard G. Parker

Acute exposure of rats, rabbits and man to moderate or severe hypoxia is accompanied by a transient abrupt rise in erythropoietin titer followed by a fall in titer to very low or undetectable levels in spite of continued exposure to hypoxia (1-4). The magnitude of the rise depends on the severity of the hypoxia (5). The time at which the highest titer is achieved is apparently proportional to the size of the animal, being reported as 12-24 hr in rats (1,2), 24-48 hr in rabbits (3), and 6 days or more in man (4). Stohlman and Brecher (2) have suggested that the secondary fall in erythropoietin may be due to increased utilization by the activated marrow; however, studies on the relation between erythropoietin titer and degree of anemia in human beings indicate that very high titers may be associated with a greatly accelerated marrow in the presence of severe anemia, suggesting that the titer is more dependent on severity of anemia than on activity of the marrow (6). It is known that the initial phases of acclimatization to hypoxia occur within the first few days, and it may be that the fall in erythropoietin titer indicates an improvement in tissue oxygenation. On this basis one would expect the erythropoietin titer to follow the signs and symptoms of hypoxia (severe at first and markedly improved after a few days).

Preliminary studies on the effects of erythropoietin administration to a normal human volunteer (7) showed that the administration of concentrated but not purified erythropoietin obtained from human urine resulted in increases in all cellular elements of the blood. The donor from whom the erythropoietin was obtained was both anemic and thrombocytopenic at the time the erythropoietin was collected and subsequently developed leucopenia (6). The possibility that the concentrate contained red-cell, platelet and leucocyte-stimulating factors must be considered. The leucocyte response could be attributed to the mixture of foreign or denatured proteins contained in the concentrate.

Clarification as to which of the observed effects should be attributed to erythropoietin *per se* might result from comparison of the response obtained with exogenous erythropoietin with that obtained from induction of high titers

of endogenous erythropoietin. It is known that following acute exposure to hypoxia or hemorrhage the erythropoietin titer rises abruptly to high levels. Because hemorrhage results in loss of all cellular elements of the blood and often is accompanied by leucocytosis and thrombocytosis (7), it was thought that exposure to hypoxic hypoxia would provide the situation most comparable to exogenous erythropoietin administration. To test correlation between signs and symptoms of tissue hypoxia and erythropoietin as well as changes in cellular elements of blood titer, a human volunteer was acutely exposed to a simulated altitude of 16,400 ft. The signs and symptoms of hypoxia were evaluated frequently until obvious improvement occurred (4 days). Erythropoietin titer of serum and urine was measured each 6-hr period during and after the 4-day period of exposure.

MATERIALS AND METHODS

The subject, a 44 year-old male in excellent physical condition, underwent decompression in a specially constructed steel chamber over a period of 10 min to an atmospheric equivalent of 16,400 ft. The atmosphere was maintained relatively constant throughout the entire 4-day period. A "lock" provided access to the chamber. The compartment occupied by the subject was equipped with a dehumidifier, and the air was periodically exchanged to eliminate CO_2 accumulation. The percentage of O_2 in the chamber was frequently monitored with a Beckman oxygen analyzer. During the time the subject was in the chamber, a complete physical examination was performed every 12 hr and partial physical examinations were performed at 6-hr intervals.

Signs of neurological hypoxia were tested in the following manner. The subject's mental acuity was tested by his ability to subtract serially 7 from 100, and to perform various multiplications and divisions. He was required to spell certain words backwards, such as "William" and "laboratory." Fine coordination was tested by rapid opposition of thumb and fingers; nose touching with the patient's eyes closed; tapping the toes alternately with his left and right foot while standing and also tapping of heels rapidly with the left and right foot while standing. The subject was required to balance on one leg with his eyes closed. Position sense in his legs was tested. The Babinsky reflex, double simultaneous stimulation, response, and the deep tendon reflexes of the achilles, patella, biceps and triceps were tested. Spirometric determinations and electrocardiographic records were obtained periodically throughout the study.

For the erythropoietin assay, blood was drawn every 6 hr, immediately centrifuged, the serum removed and stored at -5° until assayed. Urine was voided into a container immersed in an ice bath. Every 6 hr the pooled urine was removed from the decompression chamber, the volume recorded, and aliquots for the various studies removed and frozen. A small sample was saved to be tested as unmodified urine, and the majority of each sample was processed by the collodion adsorption method for concentration of urinary erythropoietin (8). All samples

were assayed for erythropoietin by the hypertransfused mouse Fe^{59} red-cell-incorporation assay of DeGowin, et al. (9). The mice were given either a half milliliter of serum or urine, or a half milliliter of the urinary concentrate from a 6-hr collection dissolved in 6.5 ml of saline.

Hematocrit determinations were done by the micro-method. Hemoglobin was determined by the cyanmethemoglobin method of Crosby, et al. (10). Reticulocytes were stained by the dry, brilliant, cresyl blue technique and counterstained with Jenner-Giemsa. Platelets and leucocytes were counted under phase microscopy using the Unopette dilution technique. Plasma-iron turnover was measured frequently during the study and plasma volume was calculated from the initial dilution of plasma-bound Fe^{59}.

Prior to, in the course of, and after the period of decompression the serum was tested for changes in circulating iodine. Total iodine was estimated directly on a 0.5 ml of serum, and protein-bound iodine (PBI) values were obtained on an equal volume of serum after precipitation by 5% trichloro-acetic acid. The micro-iodine determinations were done according to a procedure modified from Chaney (11). All determinations were performed in duplicate. Blood loss from sampling averaged 180 ml per day.

RESULTS

CLINICAL FINDINGS. Shortly after decompression of the chamber, the subject complained of being cold and had sudden violent muscular spasms primarily involving abdominal musculature. These spasms had the nature of large muscle twitches and did not have characteristics of colonic movement. During the succeeding half hour these large muscle twitches gradually subsided and the subject, who continued to complain of feeling cold, began to rest. During the next 24 hr he developed severe throbbing headache and nausea. These symptoms were only partially relieved by half-grain tablets of codeine and 10-mg tablets of compazine. During the period of 24 to 36 hr after decompression, the subject noted marked subsidence of headache and nausea. About 36 hr after having entered the chamber, the subject was relatively comfortable and remained so for the remainder of the experiment, except for intermittent headaches of a milder nature than that experienced during the initial 24 hr. During the latter part of the third day in the chamber, the subject experienced chills for a period of 3-4 hr. However, no temperature elevation was noted at this time, nor was any significant change of body temperature found during the entirety of the study.

Blood pressure did not change significantly during the study. Maximum rise of 15 mm mercury diastolic pressure was noted 6 hr after subject entered chamber and again 18 hr later. The subject's resting pulse rate was 70-72 beats/min. At 12 hr the pulse rate was still at 72 beats/min but had risen at 24 hr to 90 beats/min. It remained at this level until exit from the chamber.

During the period of hypoxia, no changes were observed in the subject's mental acuity, ability to solve arithmetic problems, spelling of words backwards or of his fine coordination and tests of the long tracts were all unchanged. The Babinsky reflex was never elicited, and double simultaneous stimulation was negative. However, within 12 hr of entering the chamber, the subject was noted to be hyper-reflexic, particularly manifesting hyperactivity of the patellar and achilles tendon reflexes. This hyper-reflexia continued, diminishing somewhat on the 3rd and 4th days of hypoxic exposure. However, only after the subject was removed from hypoxic conditions did the tendon reflexes return to normal.

After spending approximately two and a half days in the chamber, a small triangular area of the subject's left lung base exhibited fine dry expiratory rales which persisted after deep breathing and coughing. On the 3rd and 4th day of exposure the area in which these rales could be heard had extended so it now included the projection of the left posterior basal lung segment. At the end of the 4th day of hypoxic exposure, immediately on leaving the high altitude chamber, the subject had a chest X ray, including a left anterior oblique view that did not show any evidence of abnormal pulmonary densities.

Other than an increase in pulse rate, no changes in the physical examination of the heart occurred throughout the course of the experiment until the end of the 4th day. At this time a split mitral first sound was heard for the first time.

Electrocardiographic tracings were obtained every 12 hr at rest during the study. No significant changes occurred in the configuration of the tracing until two and a half days following initiation of hypoxic exposure. At this time clear-cut depression in the amplitude of the T waves were noted in the left lateral precordium and AVL. These changes progressed and were maximal at 72 hr of exposure. These changes completely reverted to the normal control (pre-exposure) pattern within the next 24 hr while still under hypoxic stress (Fig. 1).

SPIROMETRY. The control respiratory rates were 14.3 and 13.0 on the days preceding entrance into the chamber. Within 12 hr of hypoxic exposure the respiratory rate doubled. At 48 hr, respiratory rate decreased to levels somewhat higher than the control level. Respiratory rate for the remainder of the experiment was in the range of 16 to 17 per min. The oxygen consumption, corrected to standard temperature and pressure, remained relatively constant throughout the entire observation period, becoming maximally elevated on the 3rd day. Several control vital capacities gave a value of approximately 5 liters; however, during the entire period of hypoxia, the patient's vital capacity seemed to decrease approximately 20% and remained at about 4.6 liters per min. The minute-volume showed only one significant change, occurring 12 hr after beginning hypoxic

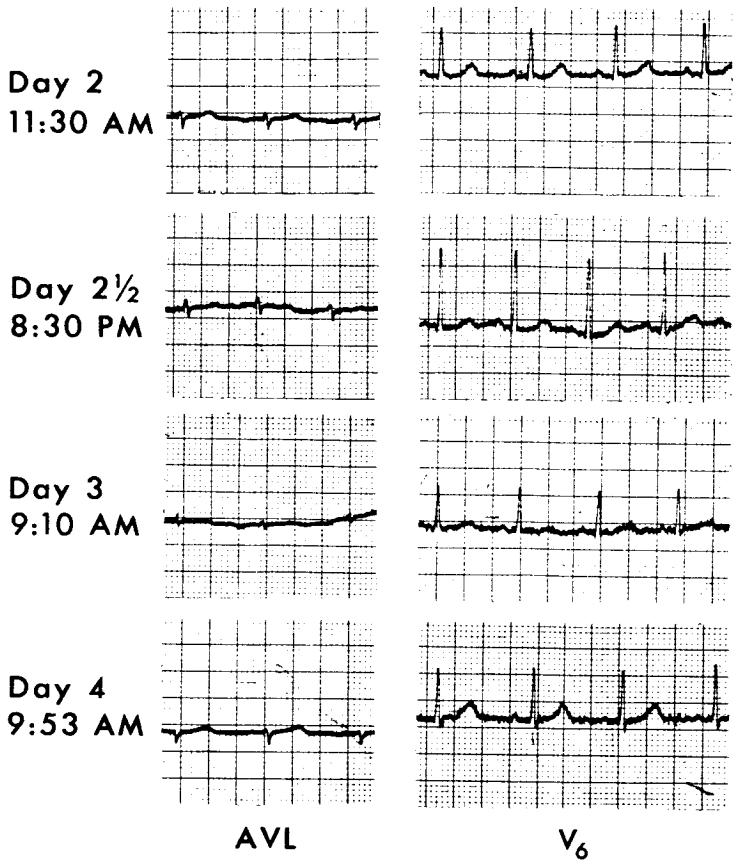

Figure 1. Progressive hypoxic changes occurring in left lateral precordium as shown in EKG leads AVL and V_6. The EKG after 2 days of hypoxic exposure is identical to that obtained prior to hypoxic exposure and markedly similar to that obtained after 4 days of hypoxic exposure. At 2 1/2 days of hypoxic exposure, T waves decrease in amplitude until the end of the 3rd day. This correlates with erythropoietin titers as shown in Fig. 2.
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exposure, when it doubled its value from the base line. The base line varied from about five to five and a half liters per minute and at 12 hr rose to 10 liters per minute attendant with the doubled respiratory rate. Within 24 hr after exposure the minute-volume dropped to control levels and remained at those levels for the remainder of the experiment. The tidal volume remained relatively constant throughout the entire course of the experiment, except for some decrease noted after 24 hr and extending up until the 3rd day of exposure at which time it returned to normal level. The maximum breathing capacity showed approximately a 20-30% increase within 24 hr after entering the chamber. This increase continued throughout the exposure to a simulated altitude of 16,400 ft. The ventilatory equivalent where both the minute-volume and the oxygen consumption were reduced to standard temperature and pressure conditions dropped to approximately half of its control base line levels at 36 hr and remained at this level for the remainder of the experiment.

BLOOD CHEMISTRY. A significant change in serum iodine was observed. Both PBI and total iodine showed a rise (Table 1) which persisted throughout the period of decompression. The increase in serum protein-bound iodine reached a peak at 48 hr, and the oxygen consumption reached a peak at 72 hr. The PBI

Table 1. Effect of decompression on serum iodine in human (μg I/100 ml)

Time relative to decompression	PBI	Iodide*	Total
24 hr	3.4	1.7	5.1
0 hr	3.7	1.7	5.4
24 hr	4.8	0.5	5.3
48 hr	6.6	0.4	7.0
72 hr	6.2	--	--
96 hr	5.7	0.4	6.1
After return to sea level conditions			
2 days	4.8	0.4	5.2
25 days	4.0	0.7	4.7

*Obtained by difference.

dropped to 4.8 γ% 2 days after subject left the chamber. Slight decrease in serum bicarbonate levels was noted during the period of chamber exposure. No significant changes were noted in the serial serum values of sodium, potassium, chloride, calcium, phosphorus, cholesterol, fasting blood sugar, uric acid, non-protein nitrogen, creatinine, bilirubin, total protein, or serum-protein electrophoretic pattern. The erythrocyte sedimentation rate was slightly increased on the 3rd day (10 mm/hr above the base line of 2-4 mm/hr).

URINE CHEMISTRIES. No consistent change occurred in the excretion of uric acid, creatinine, phosphorus, non-protein nitrogen, sodium, potassium or chloride. The creatinine clearances showed no significant or consistent change throughout the observation period. The results of the urine adrenal cortical steroid excretory patterns will be summarized elsewhere. (Siri, Cleveland, et al., to be published.)

ERYTHROPOIETIN. The results of the assays for erythropoietic activity of serum are presented in Fig. 2. Untreated mice or mice injected with saline or serum from the subject prior to exposure to hypoxia incorporated approximately 0.2% of the injected dose of Fe^{59}. The control uptake is indicated by the shaded area in the figures. From the figure it can be seen that the first sample (after 6 hr of exposure to hypoxia) was not different from the controls, but subsequent samples increased Fe^{59} incorporation with an apparent maximum during the 3rd day of exposure. Samples collected one and a half and 2 days after return to one atmosphere barometric pressure were not significantly above the control values.

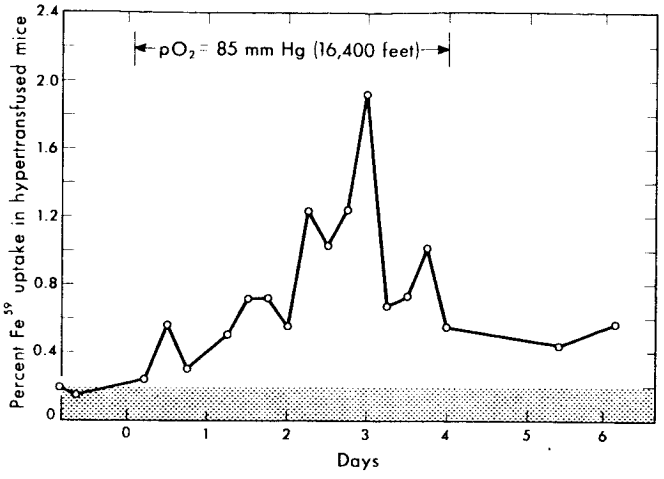

Figure 2. Serum erythropoietic activity of normal human subject before, during and following exposure to a simulated altitude of 16,400 ft for 4 days. Serum dose was 0.5 ml, and each point represents the average from 4 to 9 mice. Shaded area at the bottom of the graph indicates the maximum value obtained in controls who received serum from the subject prior to exposure to hypoxia or saline.

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By comparison to a dose-response curve prepared with human urinary erythropoietin previously compared to the C.S. (Comparative Standard) (12), it is estimated that the maximum value obtained was equivalent to approximately 0.14 C.S. units/ml serum.

No erythropoietic activity was detected in unmodified urine. The results obtained with urinary concentrates are shown in Fig. 3. Definite activity can be seen in most samples collected after 24 hr, with an apparent maximum at 48 hr of exposure to hypoxia. One sample collected 2 days after return to sea-level conditions gave a result identical to the pre-exposure sample, neither being significantly higher than the saline-injected controls.

PERIPHERAL BLOOD. The results of serial hemoglobin, hematocrit, and plasma-volume determinations are shown in Fig. 4. As can be seen from the figure, hemoglobin and hematocrit remained fairly stable during the period of exposure to reduced atmospheric pressure and fell 20% following return to sea-level conditions. The plasma volume showed a small progressive rise throughout the period of observation.

The values for the concentration of leucocytes, platelets, and reticulocytes are plotted in Fig. 5. An abrupt rise in total leucocyte count to approximately twice the pre-treatment level is seen soon after onset of exposure, followed by an irregular return to the pre-treatment level during the subsequent 2 to 3 weeks. The platelet count rose gradually during the period of exposure and remained elevated above the pre-treatment level for a period of approximately 3 weeks. A wave of reticulocytes was observed with a maximum 7 days after the onset of exposure to hypoxia. The proportion of granulocytes and lymphocytes remained unchanged throughout the study (40% lymphocytes), i.e. the changes in leucocyte count were due equally to granulocytes and lymphocytes. Eosinophils (4% before exposure) fell to 1% by 6 hr and remained at that level for 10 days following onset of hypoxia, after which they returned to the pre-treatment level.

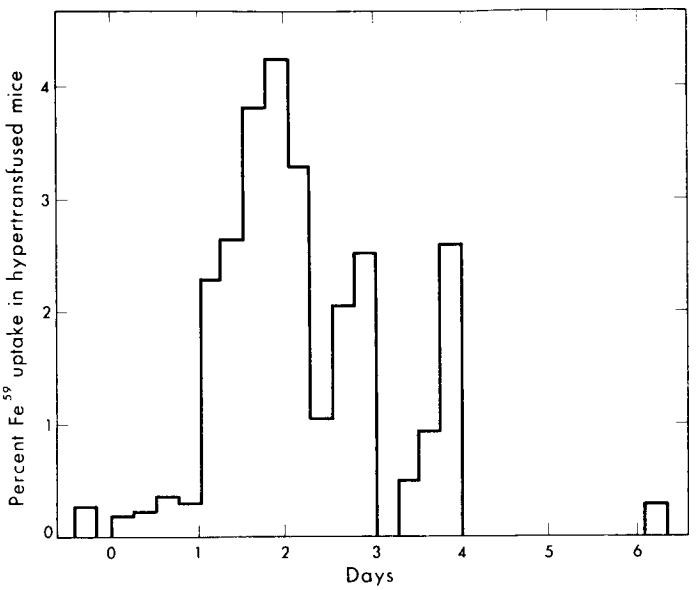

Figure 3. Erythropoietic activity of urine extracts before, during and after exposure to hypoxia. A 6 hr collection of urine was extracted by the collodion adsorption method. The extract was dissolved in 6 ml saline and a single injection of 1/2 ml given each mouse. Each value in the figure is the average of 6-10 mice.

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IRON KINETICS AND HEMOGLOBIN SYNTHESIS. The results of the plasma-iron-turnover determinations taken during the study are presented in Table 2. Approximately 1 hr after acute exposure to an oxygen pressure equivalent to that present at an altitude of 16,400 ft, despite a decrease of plasma-iron concentration from 112 μg/100 ml to 76 μg/100 ml, the plasma-iron turnover was essentially unchanged at 1.04 mg/hr. Eleven hours later a distinct increase was noted. Plasma-iron turnover continued to increase progressively until a maximum value of 2.70 mg/hr was reached two and a half days after onset of hypoxia. The plasma-iron concentration remained rather constant during this period at approximately 96 μg/100 ml. During the subsequent day and a half ending the 4-day period of hypoxia, the plasma-iron concentration decreased to approximately 50 μg/100 ml, and the plasma-iron turnover decreased to approximately 2.03 mg/hr. Subsequently, plasma-iron turnover decreased slightly during the first two days after return to sea-level oxygen pressure, after which a more rapid decline toward the initial baseline value took place.

Increased values of plasma-iron turnover are only roughly correlated with erythropoiesis, and plasma iron turnover may be considerably increased, as in patients with endogenous hemochromatosis, without any increase of erythropoiesis (13). In order to obtain a better estimation of erythropoiesis throughout the study, the rates of hemoglobin synthesis were calculated using the following assumptions: 1) The initial (baseline) rate of hemoglobin synthesis is equal to total circulating hemoglobin/mean erythrocyte survival = 564 g/117 day = 4.82 g/day. Total circulating hemoglobin is obtained from measured red-cell volume 1830 ml and mean corpuscular hemoglobin concentration 0.31; erythrocyte survival is assumed to be 117 days, the mean value obtained from complete iron-kinetic studies of 13 normal subjects (13). The fraction of plasma-iron turnover irreversibly fixed for hemoglobin synthesis is in this way calculated to be

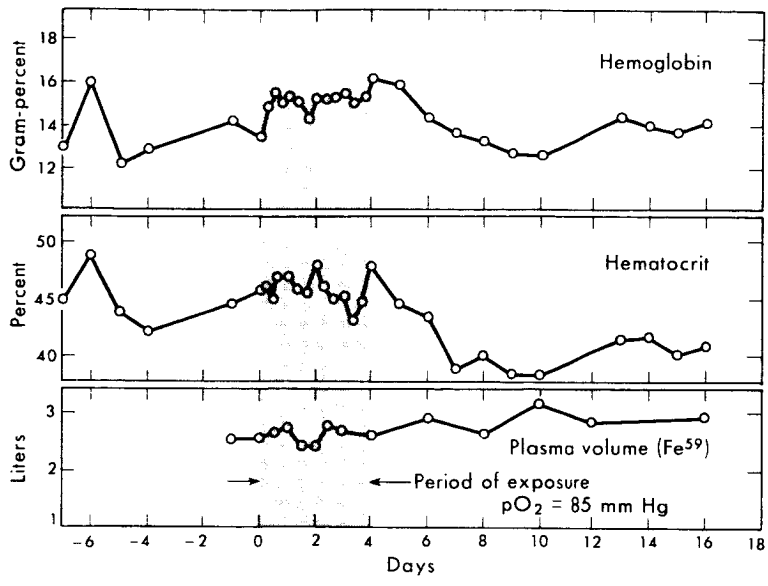

Figure 4. Comparison of the changes in hemoglobin, hematocrit and plasma volume before, during and after exposure to a simulated altitude of 16,400 ft (the shaded area). Plasma volume was determined by plasma bound Fe^{59} dilution.

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$$\frac{4.82 \text{ g Hb/day} \times 3.4 \text{ mg Fe/g Hb}}{25.1 \text{ mg Fe/day}} = \frac{16.4 \text{ mg Fe/day}}{25.1 \text{ mg Fe/day}} = 0.65$$

This is in excellent agreement with the corresponding mean fraction of 0.66 (21.4/32.5) obtained from complete iron-kinetic studies of 13 normal subjects (13). 2) Subsequent rates of hemoglobin synthesis are calculated by using this fraction (0.65) of plasma-iron turnover whenever the plasma-iron concentration exceeds 92 μg/100 ml and by using a fraction of 0.95 when the plasma-iron concentration reached a minimum of 53 μg/100 ml. Previous iron-kinetic studies of patients with low plasma-iron concentrations have demonstrated this increased fraction of plasma-iron turnover that is irreversibly fixed for hemoglobin synthesis (13). For values of plasma-iron concentration between 53 μg/100 ml and 93 μg/100 ml, corresponding fractions are interpolated between 0.95 and 0.65. In this way, measurements of plasma-iron turnover are used to obtain calculated rates of iron incorporation into hemoglobin and, hence, the rates of hemoglobin synthesis per hour (3.4 mg Fe per gram hemoglobin) as shown in Table 2.

Hemoglobin synthesis is definitely increased 11 hr after the onset of hypoxia and continues to increase until approximately one day after return to sea-level atmospheric pressure. Between the second and fourth days after return to sea-level conditions the rate of hemoglobin synthesis decreased rapidly, after which a more gradual decrease towards its initial rate occurred.

DISCUSSION

Since the method of extraction of the urine cannot be assumed to give uniform recovery of erythropoietin from all samples, no conclusions as to the exact time of maximum urinary activity or as to differences between the urinary and serum titers should be drawn.

The plasma erythropoietin titer rose initially to a maximum on the third

Table 2. Calculation of the rates of hemoglobin synthesis from plasma iron turnover measurements

		Plasma Fe turnover mg/hr	Fraction of plasma Fe turnover used for Hb synthesis	Fe for Hb synthesis mg/hr	Hb synthesis g/hr
	10/22 AM	1.035	.654	.676	.199
HYPOXIA	10/23 AM	1.050	.654	.686	.202
	PM	1.466	.654	.958	.282
	10/24 AM	1.62	.654	1.059	.312
	PM	1.834	.654	1.200	.353
	10/25 AM	2.013	.654	1.315	.387
	PM	2.70	.654	1.764	.519
	10/26 AM	2.228	.827	1.840	.541
	10/27 AM	2.03	.954	1.940	.570
	10/28	2.02	.954	1.93	.568
	10/29 AM	1.877	.954	1.790	.526
	10/31 AM	1.548	.714	1.100	.324
	11/2 AM	1.48	.654	0.968	.285
	11/4 AM	1.514	.654	0.990	.291
	11/8 AM	1.344	.654	0.88	.259

day of exposure and then fell. One possible explanation for the erythropoietin titer rise and fall so soon after the onset of hypoxia is that the subject is more hypoxic during the first day or two at a given "altitude." The "initial acclimatization" occurs within the first few days (cardiac and respiratory adjustment) so that tissue oxygenation is significantly improved by the 3rd or 4th day.

Twenty-four hours after initial exposure the subject first showed clinical evidence of acclimatization to hypoxic hypoxia. Subsidence of nausea, increase in appetite and marked diminution in headache occurred. The subject dramatically increased his efficiency in extracting oxygen from the inspired air, as evidenced by marked decrease in minute-volume while his oxygen consumption remained constant.

It is noteworthy that after 24 hr of chamber exposure, when the subject

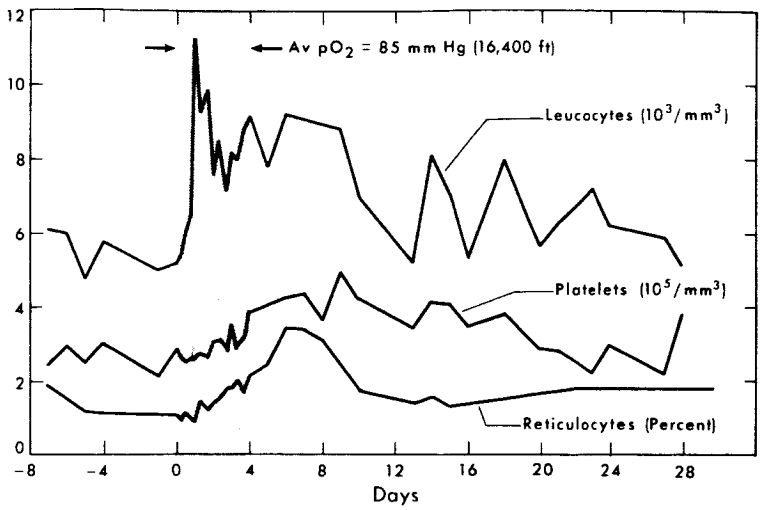

Figure 5. Results of serial leucocyte, platelet and reticulocyte counts before, during and following exposure to a simulated altitude of 16,400 ft (the shaded area).

MU-30141

clinically felt much improved and relieved symptomatically, the pulse rate increased from a base line in the low 70's to 90 beats per minute, the respiratory rate dropped to levels somewhat higher than that of the base line, the minute-volume decreased to base line values, and the efficiency of oxygen extraction doubled, which suggests that acclimatization to hypoxia is closely related to the ability of the lungs and body tissue to extract oxygen more efficiently from the inspired air. The elevation of maximum breathing capacity probably resulted from the decreased viscosity of air at diminished atmospheric pressure.

This first evidence of acclimatization coincides with the maximum urine erythropoietin titers (24 hr). Following this, after 24 hr, there was continuing gradual improvement in the subject's symptoms and mental outlook until the cessation of hypoxia. The electrocardiogram revealed another facet of the subject's physiological acclimatization: after 48 hr of hypoxic hypoxia no change had occurred in the electrocardiographic pattern relative to tracings obtained prior to initiation of hypoxia. During the period of 48-72 hr, progressive evidence for myocardial hypoxia appeared, as evidenced by decreasing amplitude of the T waves in the left lateral precordial leads and AVL (Fig. 1). This corresponded in timing exactly to the elevations in serum erythropoietin (Fig. 2).

Thus, a good correlation was established between degree of hypoxia and the erythropoietin titer. These observations suggest that the fall in erythropoietin is probably a reflection of improvement in tissue oxygenation due to the initial acclimatization process. However, they do not rule out the suggestion of Stohlman and Brecher (2) that the fall is a result of increased utilization of erythropoietin by the marrow.

Scaro (4), in a study on the erythropoietin content of urine pooled from nine subjects as a function of time after exposure to hypoxia (at 3,990 meters), found a progressive increase in titer from the 2nd through the 6th, and last,

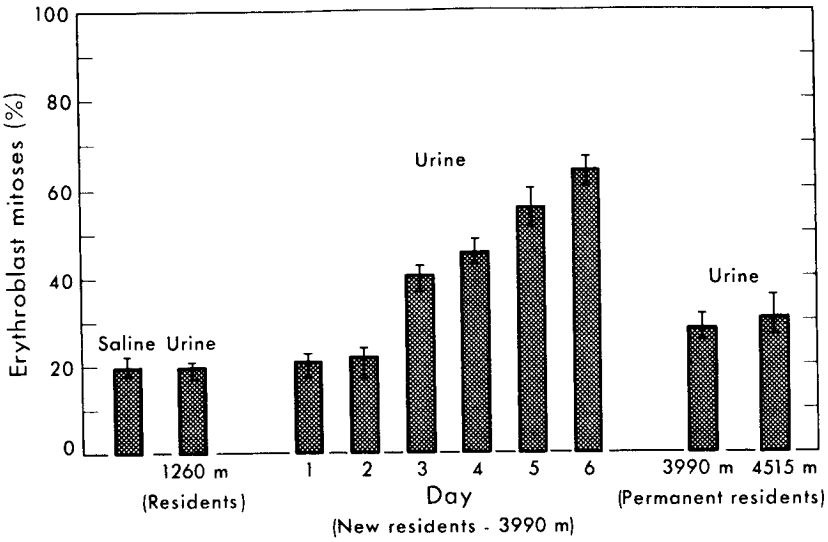

Figure 6. Increase in proliferation of erythroblasts produced in the bone marrow of rats by the injection of alcohol extracts of human urine. Translated from Scaro (4). MU-30142

day of the study. A much lower but significantly positive effect was found from the urine of long term residents at the same altitude, Fig. 6. The data presented are in agreement with the results of the present study except for the fact that Scaro found the fall in titer occurred sometime after 6 days, whereas in the present study the urine titer had begun to fall by the 3rd day. This difference may be the result of variation in the time required for initial acclimatization in different subjects.

A second object of this study was to compare the changes in formed elements of the blood previously observed to occur with exogenous erythropoietin administration (13) to the changes occurring during a period of high endogenous erythropoietin production (acute hypoxic hypoxia). In both a previous subject given erythropoietin and in this subject, in whom a high titer of erythropoietin was demonstrated during acute hypoxia, there was an increase in the concentration of all formed elements of the blood. This increase could not be accounted for on the basis of hemoconcentration. The results of the two studies have been superimposed in Fig. 7-9. The time of onset, duration and relative magnitude of the changes were strikingly similar in the two subjects although the increases were more marked in the subject given large doses of erythropoietin for a period of 7 days than in the present subject exposed to hypoxia for 4 days. (Fig. 10)

It is realized that the evidence presented is inadequate to support any conclusions as to the exact site of action of what has been called "erythropoietin." Whether one is observing multiple effects of a single substance (hemopoietin) or multiple factors (erythropoietin, thrombopoietin, granulopoietin, and lymphopoietin), or a combination of such factors released in response to hypoxia or anemia is not apparent.

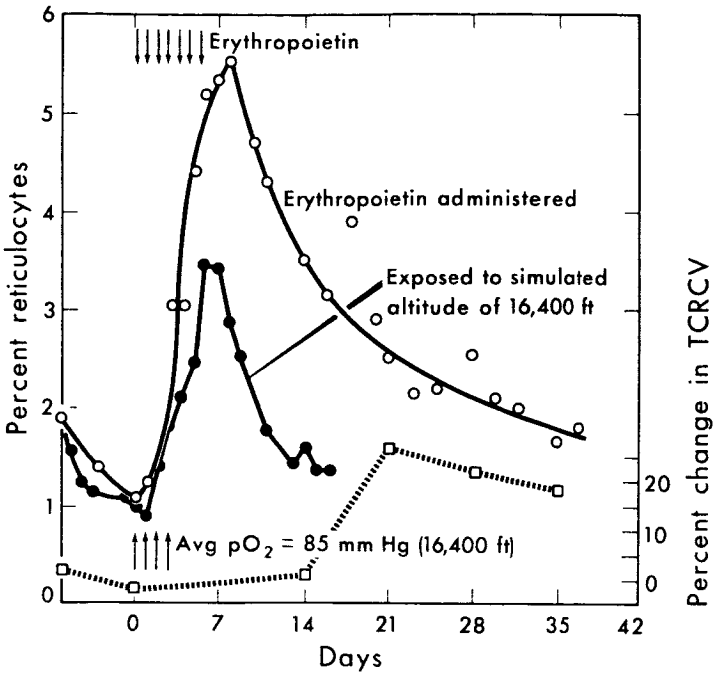

Figure 7. Comparison of the changes in reticulocyte count of a normal subject given human urinary erythropoietin for 7 days with the reticulocyte count of a normal subject exposed to a simulated altitude of 16,400 ft for 4 days. The broken line at the bottom of the figure shows the percent change in total circulating red-cell volume of the erythropoietin-treated subject. Figs. 7, 8 and 9 are the same subjects.

MU-30143

Previous tracer studies of plasma-radioiron removal in two normal subjects subjected for 8 hr to an oxygen pressure corresponding to 14,500 ft demonstrated no increase of plasma-iron turnover during the 8-hr period. Plasma-iron turnover and hemoglobin synthesis were distinctly elevated at 11 hr in this subject suggesting that an increase of erythrocyte precursors in the marrow, probably proerythroblasts, occurs somewhat between 8 and 11 hr after the acute onset of hypoxia. Hemoglobin synthesis and erythrocyte precursors continued to increase throughout the entire 4-day period of hypoxia. The rate of increase of erythropoiesis was maximal between one and a half days and two and a half days after the onset of hypoxia. This would correspond to the time interval, 1-2 days after proerythroblast formation, at which time mitotic divisions give rise to erythroblasts. Erythrocyte precursors appeared maximal approximately one-half day after cessation of hypoxia, approximately 2 days after maximal plasma-erythropoietin concentration occurred. Maximal hemoglobin synthesis was almost three times normal approximately 2 days after the maximal erythropoietin concentration was reached, which was approximately 5 days after onset of hypoxia.

SUMMARY

A normal subject, acutely exposed to a simulated altitude of 16,400 ft, showed evidence of significant acclimatization within a period of 4 days. Signs and symptoms of hypoxia, severe at first, had largely disappeared by the 4th day. Serial electrocardiograms showed evidence of myocardial hypoxia (flattened T waves) which was most marked at the beginning of the 3rd day. By the end of the 4th day the electrocardiography had returned to normal. The erythropoietin titer of the serum paralleled the electrocardiographic changes, the highest titer occuring at the beginning of the 3rd day.

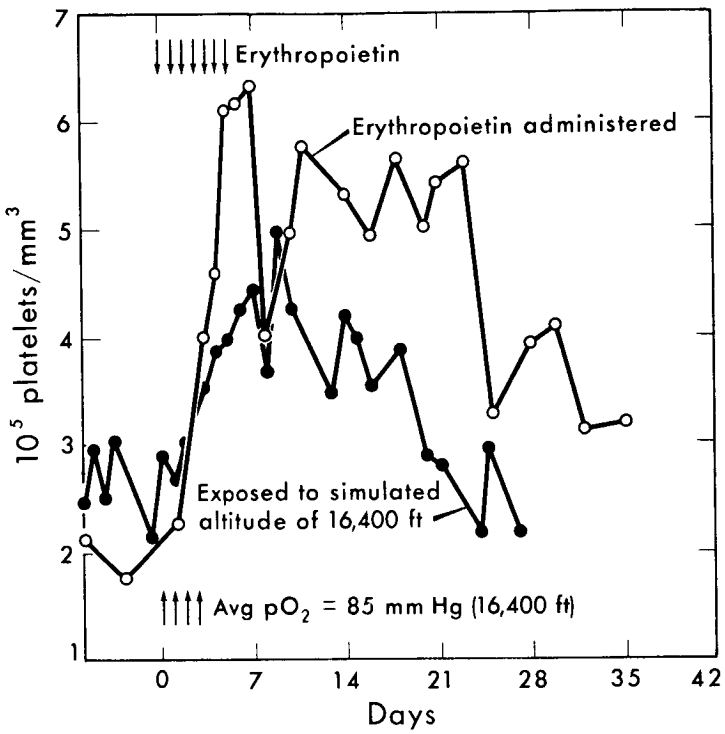

Figure 8. Comparison of the changes in platelet count in a normal subject given human urinary erythropoietin for 7 days with the platelet count of a normal subject exposed to a simulated altitude of 16,400 ft for 4 days.

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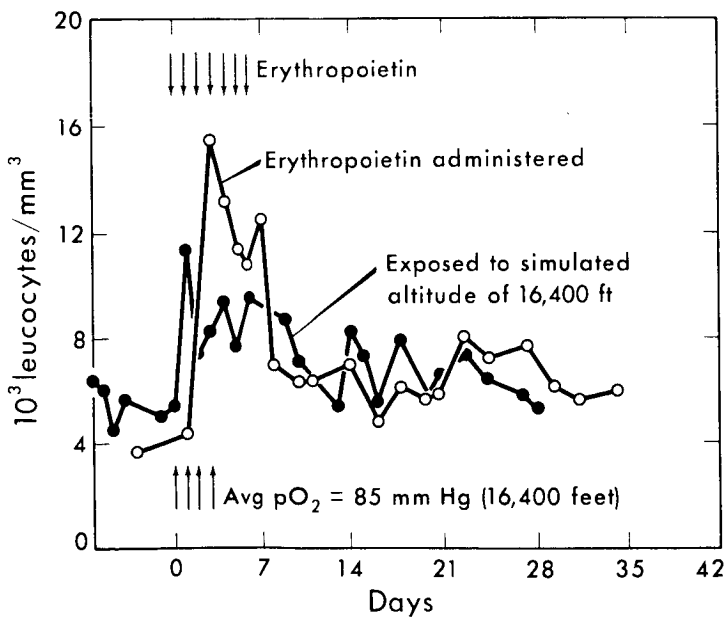

Figure 9. Comparison of the changes in total leucocyte count of a normal subject given human urinary erythropoietin for 7 days with the leucocyte count of a normal subject exposed to a simulated altitude of 16,400 ft for 4 days.

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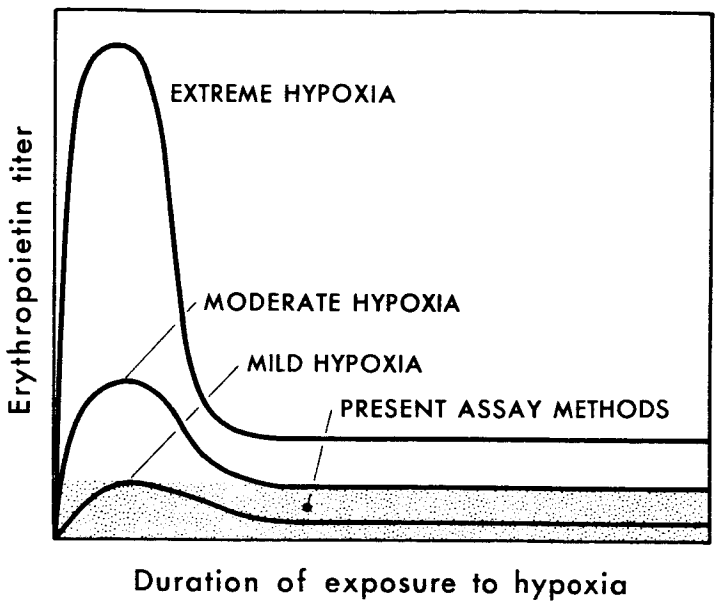

Figure 10. Possible relation between duration of exposure to hypoxia and erythropoietin titer. The shaded area at the bottom of the figure indicates the lower limit of sensitivity of present assay methods. (Reproduced from Van Dyke, Lawrence and Siri, Acta Isotopica 3: 217, 1961)

MU-25575

These findings suggest that the abrupt rise and fall in erythropoietin titer characteristic of acute exposure to hypoxia are the result of initial tissue hypoxia followed by rapid improvement in tissue oxygenation resulting from the initial acclimatization process.

Increase and subsequent decrease of hemoglobin synthesis occurred after the corresponding changes in erythropoietin titers; the onset of increased hemoglobin synthesis occurred after 8 hr with maximal hemoglobin synthesis taking place during this experiment approximately 48 hr after the peak erythropoietin titer.

ACKNOWLEDGEMENTS

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Gradient-Elution Chromatography of Rat-Brain-Lipid Extracts

Gary J. Nelson

The complexity of brain lipid extracts has greatly added to the difficulty of separating the components into pure fractions. In the past, successful isolation of pure lipids from brain tissue has usually involved tedieus solvent fractionation or partial hydrolytic methods, while the relatively simple technique of column chromatography frequently has yielded poor results. However, recent studies with column methods indicate that this technique may be useful in brain lipid studies, particularly when the fatty-acid composition of the various fractions is being investigated. Alumina has been used for partial fractionation followed by further manipulations by Long and Staples (1) and Davison and Wajda (2). The resolution of alumina columns is not good, however. Biran and Bartley (3) reported the separation of total-brain-lipid mixture on silicic-acid columns. However, they analyzed only the phospholipids and neglected the cerebrosides. Recently Faillace and Bogoch (4) described an attempt to separate total-brain lipid extract by stepwise silicic-acid column chromatography and reported the isolation of an aminoglycolipid in this manner. Earlier, Weiss (5) separated brain and spinal-cord cerebrosides with a gradient-elution procedure on silicic acid. In more recent work Schwarz et al. (6) also fractionated sphingolipids from human brain on a silicic-acid column and investigated the fatty-acid composition of the separated components.

In the following paper some initial studies on the fractionation of total lipid extracts of normal whole rat brain by silicic-acid-column chromatography, using a newly developed method (7) of concave-gradient elution with methanol in chloroform, are described. Analyses of the various fractions and fatty-acid composition as determined by gas chromatography are reported.

MATERIALS AND METHODS

Normal male rats of the Wistar strain, approximately 6 months old were used in this study. The animals had received a laboratory feed ad libitum since weaning. They were lightly anesthesized and then sacrificed by decapitation and the brain was removed and processed immediately. The whole brain was weighed and then washed in physiological saline. It was homogenized in 160 ml of methanol-chloroform 1 to 1 (V/V) for 2 min in a blender. The homogenate was transferred to a 250-ml volumetric flask and heated at 60° for 15 min, the flask was cooled and brought to volume with chloroform. The protein

residue was removed by filtration through a fat-free and pre-washed filter paper. After the recovery was measured, the solvent was transferred to centrifugable separatory funnels (125-ml size) and 20% distilled H_2O by volume was added to each. They were shaken vigorously for 5 min, and the resulting emulsion was broken by centrifuging for 15 min at 1000 rpm. The organic layers from each separatory funnel were combined in a 250-ml round-bottom flask, and the solvent was removed under reduced pressure at 45° in a rotary evaporator. The dry lipid residue was redissolved in a small volume of chloroform (about 5 ml) and transferred to a previously tared vial, taken to dryness under N_2 at 60° and placed in a vacuum desiccator for a minimum of 18 hr. Then the sample was weighed, dissolved in CCl_4 and transferred to a 10-ml volumetric flask. Portions of the sample were taken for the analyses described below, and the remainder was used for the column chromatography.

CHROMATOGRAPHY. The column, prepared as described elsewhere (7), contained 20 g of silicic acid (100-325 mesh), and was 18 × 140 mm. The lipid charge was added to the column in carbon tetrachloride. The loading factor was 6 to 7 mg total lipid extract per g of packing. Elution was begun with chloroform and continued until 1000 ml of this solvent had passed through the column.

In previous experiments (8) pure chloroform was found to elute only relatively non-polar lipid, such as triglycerides and cholesterol, while phospholipids and cerebrosides remained on the column. Cholesterol was the only lipid expected in this fraction, so the entire volume (1000 ml) was collected as a single fraction. The chloroform was removed by low-pressure distillation and the lipid residue dried, weighed, and stored at 4° under N_2 until further analysis.

Immediately after the chloroform elution had finished, gradient elution was begun. The apparatus and details for the production of the gradients used in this work have been described in another publication (7). The flow rate through the column was maintained at 1 ml/min by constant-volume pumps. Fractions containing 10 ml of eluted solvent were collected during the course of the gradient elution automatically by a constant-time-indexed automatic fraction collector while the gradient varied from zero to 100% methanol in chloroform. At the conclusion of the gradient elution an additional 300 ml of methanol containing 5 % distilled H_2O was passed through the column. The eluate was collected in 16 × 150-mm screw-cap test tubes with Teflon-lined caps. All tubes were capped as quickly as possible after being filled, and stored at low temperature until further analysis.

ANALYTICAL PROCEDURES. The total lipid extract was subjected to tests for sulphate, sphingosine, phosphorus, galactose, free amino groups, and to infrared analysis, and transmethylation for analysis of fatty acids by gas chromatography. A portion of the neutral lipid-fraction was rechromatographed

on a small silicic-acid column (9) and the fractions analyzed for triglycerides and cholesterol esters by infrared spectroscopy (10). Phosphorus determinations were performed on 0.5-ml aliquots by the method described previously (11). Galactose analyses were performed by the anthrone method of Radin et al. (12), on 1.0-ml aliquots from the various collection tubes.

Sulphate was estimated by a modification of barium-chloranilate procedure of Bertolacini and Barney (13). Briefly, the sample was first hydrolyzed in 1 N HCl at 90° C for 2 hr. Then 2.5 ml of pH 4 acetate buffer, 7 ml of isopropyl alcohol, and 5 mg of Ba chloranilate were added, and the tube was shaken intermittently for 3 min, centrifuged, and the optical density of the supernatant read at 320 mμ and compared with a standard curve prepared in a similar manner.

These tests defined the elution pattern shown in Fig. 1. Using this information the various peaks were determined and the collection tubes combined into fractions as indicated in Table 3. Infrared spectra of the combined fractions were determined, and analyses were then performed on the material isolated from each fraction. Sphingosine was analyzed by the method described previously (7). Plasmalogens were not analyzed quantitatively but were qualitatively estimated from the recovery of fatty-aldehyde dimethyl acetals after transmethylation of the various fractions.

Thin-layer silicic-acid chromatography was performed on the combined fractions. Plates were prepared as described elsewhere (7). Solvent systems of varying proportions of methanol in chloroform-H_2O were used, so that each fraction was chromatographed with a solvent which had the approximate composition of the solvent that eluted the fraction from the silicic-acid column. Spots were developed either by charring with concentrated H_2SO_4 or by spraying the plate with 2', 7', di-chlorofluorescein and viewing under ultraviolet light. In addition, unsaturated spots were detected by exposing plates to iodine vapors and free amino groups by spraying with ninhydrin in acetone-lutidine solution.

The fatty acids were recovered by transmethylating (8) suitable amounts from the various peaks and extracting the methyl esters in hexane to determine percentage recoveries from each fraction. The fatty-acid composition of each fraction was analyzed by gas chromatography as previously described (8) with the equipment and methods reported in detail elsewhere (14, 15). Methyl-ester samples were hydrogenated (16) and the results of the gas chromatography of the hydrogenated and non-hydrogenated samples were compared to identify the various components. Fatty-aldehyde dimethyl acetals were not separated from the fatty-acid methyl esters before gas chromatography. However, it was possible to analyze both aldehydes and acids on the same diethylene-glycol-succinate column to a considerable degree (17). In certain cases unsaturated fatty acids had retention times identical to saturated methyl esters of different chain

length and it was not possible to obtain separation of the individual components.

Hydroxy-fatty-acid esters were separated from the normal fatty acids by the Florisil-column chromatographic method developed by Kishimoto and Radin (18). The separated fractions were quantitatively analyzed by infrared spectrophotometric methods (9, 10) and the hydroxy fatty acids identified by their characteristic hydroxyl absorption at 2.8 μ.

RESULTS

In an earlier report (19) it was indicated that some lipid was not removed from the tissue by the extraction procedure. Recent studies, however, have shown that the method used in this and the earlier work is complete; i.e., no lipid could be recovered from the insoluble residue beyond what was expected from solvent retention.

Table 1 summarizes the results of a typical extraction of a normal rat brain. Only 3.1 mg of chloroform-soluble material was recovered from the digestion of the proteinaceous residue with KOH (1N) for 2 days at 60° under N_2. From the volume of organic solvent remaining in the residue approximately 2.5 mg of material were expected here. The extraction was considered complete.

Table 2 gives the results of analyses on the total lipid extract for the various components and the recovery of material from the silicic-acid column, while Table 3 presents a detailed analysis of the chromatographic fractions from the run shown in Fig. 1. Fractions 1, 5, 6, 8, 9, and 10 contained essentially all of the phosphorus applied to the column, while fractions 2, 3, 4, and 7 contained essentially all of the galactose. Fractions 1 and 2 contained only a small fraction of the material applied to the column and were not identified. Fraction 3 is cerebroside. It had a galactose-to-sphingosine ratio of 0.91 and its infrared spectrum agreed with that of a known phrenosine sample. Fraction 4 contained the largest amount of galactose and had a sphingosine-to-galactose ratio of 0.87. However, the total percentage of galactose was below that found in fraction 3. The infrared spectra of this fraction shown in Fig. 2 was quite different from fraction 3. Its spectrum indicates that there is no ester absorption but considerable amide and hexose absorption. There appears to be more amide absorption in this compound than can be accounted for by the amino group of sphingosine; hence, this material may contain hexose amine. The data of Weiss (5) also indicated excess nitrogen in this fraction.

Fraction 5 was not definitely identified. It had a phosphate-to-fatty-acid ratio of 1.94 and an infrared spectrum similar in some respects to phosphatidyl ethanolamine yet different enough to rule out identity. As this fraction was not completely resolved from fraction 5, it was probably a mixture of

Table 1. Extraction of whole rat brain by methanol-chloroform

Fraction	Weight in mg %	% Wet weight
1. Wet weight of tissue	1629.0	-
2. Chloroform-methanol extract	204.5	12.6
3. Total residue in aqueous phase	41.7	2.6
4. Chloroform-soluble residue in aqueous phase	1.4	0.1
5. Lipid recovered from protein residue after digestion with 1N KOH	3.1	0.2

Table 2. Analysis of methanol-chloroform extract of whole rat brain and recovery from silicic-acid column

Compound	Weight, mg	Recovery, %
Total weight	139.0	105
Cholesterol	24.5	110
Phosphorus	3.18	98
Galactose	4.56	103
Sulphate	0.37	90
Sphingosine	8.85	97
Fatty acid and fatty aldehydes	66.7	100

the main component with phosphatidyl ethanolamine from fraction 6. It also contained appreciable quantity of plasmalogens, although less than the adjacent fraction 6. It gave a positive test for free amino groups. This fraction was probably phosphatidyl serine and serine plasmalogen.

Fraction 6 appeared to be largely phosphatidyl ethanolamine and its plasmalogen analogue. The infrared spectrum was typical, and the material gave a positive test for free amino groups. This fraction was slightly contaminated by the adjacent fractions. Fraction 7, while considerably overlapped by fraction 6, was primarily cerebron sulphate on the basis of its galactose, sphingosine and sulphate content as well as its infrared spectrum. Fraction 8 was probably lysophosphatidyl ethanolamine. It had a positive ninhydrin reaction and a phosphate-to-fatty-acid ratio of 1.17. Its infrared spectrum, while not compared to a standard was consistent with this assumption. No galactose or sulphate were found in this fraction. A small amount of monophosphoinositol could have been present but less than 5% of the total material in the fraction. Fraction 9 was phosphatidyl choline. Its infrared spectrum was identical to pure synthetic phosphatidyl choline except for differences expected by the

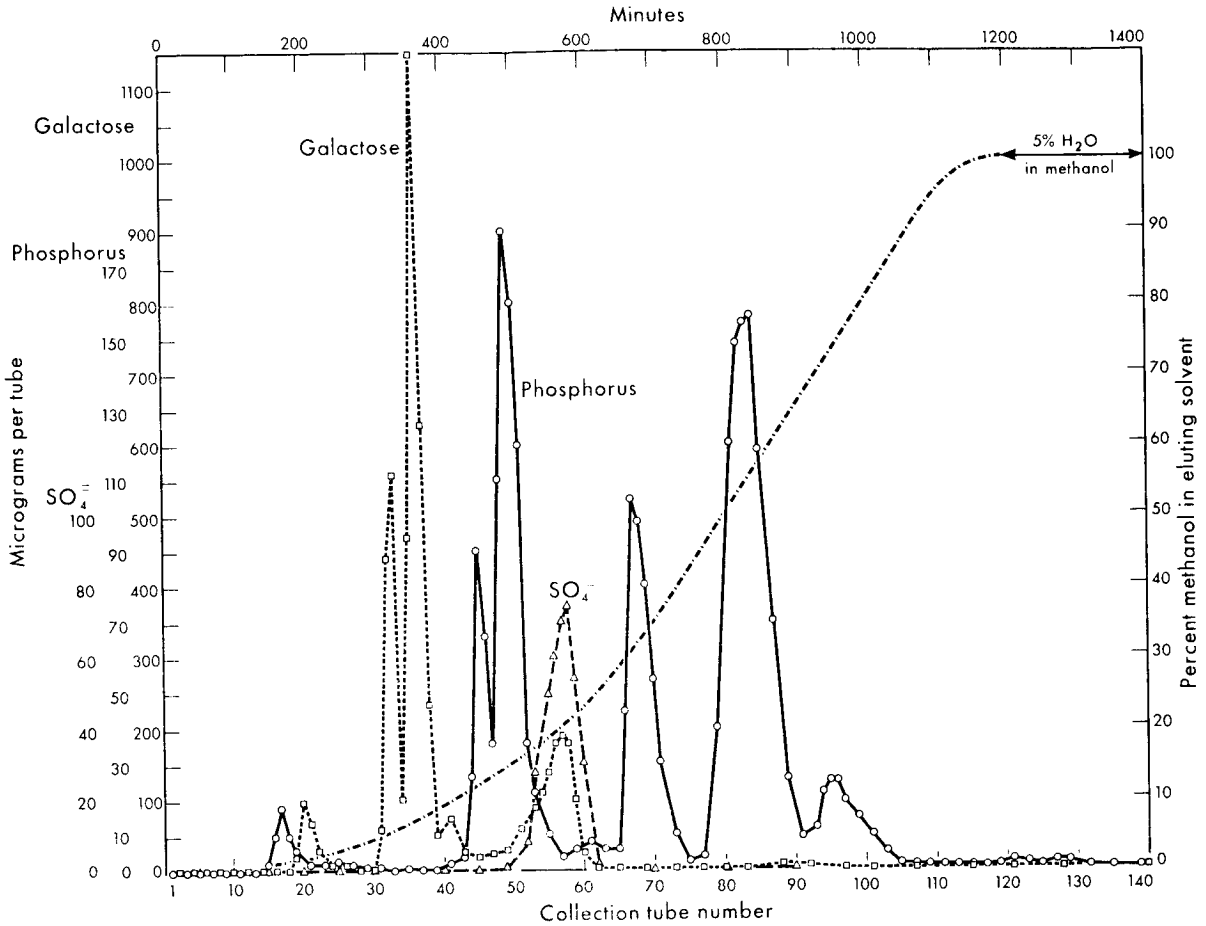

Figure 1. The elution pattern of lipids extracted from whole rat brain. Gradient elution with methanol in chloroform on silicic-acid column. Neutral lipids were removed from column previously by elution with chloroform. Weight placed on column 139 mg; recovered 146 mg. See text for details of method. MUB 1349.

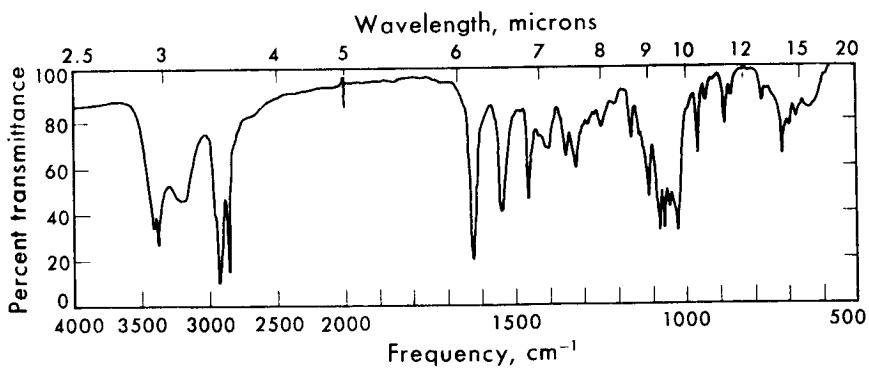

Figure 2. The infrared spectra of a solid film of the material recovered from collection tubes 34 through 42 in Fig. 1. Spectrum recorded on a Perkin-Elmer Model 421 grating infrared spectrophotometer. MU 28193.

variation in fatty-acid composition. The phosphate-to-fatty-acid ratio was 1.93, and no ninhydrin-positive substances, galactose, or sulphate were present. This fraction was the major phospholipid found in brain in all samples analyzed in detail. The percentage of the total phosphorus was 39.6, 39.5, 38.7 in three different animals. Very little choline plasmalogen was detected in this fraction.

Fraction 10 was primarily sphingomyelin on the basis of its infrared spectrum, phosphorus, and sphingosine content. Some lecithin tailed into this fraction. This fraction accounted for 5% of the total phosphorus applied to the column. No galactose or sulphate were detected in it.

No definite elution peaks were detected after sphingomyelin in any of the chromatographic runs, although a small tailing of phosphorus did occur. No lysolecithin was detected or any di- or tri-phosphoinositol. These latter compounds are known not to elute from silicic acid with methanol (Dr. C. E. Ballou, personal communication) and are lost in the aqueous wash of the organic extract.

The chloroform eluate contained no detectable free fatty acid or cholesterol ester. It did however contain a small amount (2% of the total fraction) of an unidentified hydrocarbon and about the same amount of triglyceride. The remaining substance was free cholesterol or closely related isomers as analyzed by infrared spectrophotometry.

Table 4 reports the nonhydroxy-fatty-acid composition of total-brain-lipid extracts. The analysis is the average of three brain samples run in duplicate. The hydroxy fatty acids were not eluted from the diethylene-glycol-succinate column used in this work and hence are reported as percentage of the total fatty acid. Fatty aldehydes resolved in this system are reported in Tables 4 and 6. As certain methyl esters of unsaturated fatty acid elute off the column at the same point as other saturated-fatty-acid methyl esters, combined values are reported in these cases, unless subsequent hydrogenation was able to resolve them. An asterisk is used to indicate tentative identification of certain of the elution peaks from the gas chromatographic column.

The percentage of hydroxy fatty acids in whole rat brain and the chromatographic fractions is presented in Table 5. The hydroxy acids were concentrated exclusively in the hexose sphingolipids fractions, although the amounts in the different fractions varied considerably. No hydroxy acids were detected in the glycerol phospholipids or in the sphingomyelin fraction.

Table 6 gives the fatty-acid and fatty-aldehyde composition of fractions obtained from the gradient-elution silicic-acid-column chromatography shown in Fig. 1, excluding the hydroxy fatty acids. The non-choline glycerol phospholipids contained large amounts of stearic and higher chain polyunsaturated fatty

Table 4. Nonhydroxy-fatty-acid and fatty-aldehyde composition of whole rat brain - average of three analyses (Mass % methyl esters recovered)

Retention time relative to methyl stearate	Fatty-acid ester or dimethyl acetal (DMA)	Average value	Range
0.10	10:0	0.04	0.02 - 0.07
0.17	12:0	0.08	0.04 - 0.13
0.25	14:0 DMA*	0.96	0.52 - 1.62
0.30	14:0	0.86	0.74 - 0.97
0.41	15:0	0.13	0.04 - 0.19
0.45	16:0 DMA	1.3	0.65 - 1.5
0.55	16:0 + 16:1 DMA	19.9	18.5 - 20.8
0.64	16:1	1.6	0.47 - 2.8
0.75	17:0	0.39	0.14 - 0.45
0.85	18:0 DMA	0.17	0.10 - 0.29
1.00	18:0	19.3	18.5 - 20.8
1.14	18:1	21.5	18.8 - 23.4
1.41	18:2 + 19:0	0.85	0.61 - 0.96
1.79	20.0	0.79	0.52 - 0.86
2.03	20.1*	3.4	2.8 - 3.7
2.38	21:0	0.14	0.04 - 0.30
2.88	22:0 DMA*	0.29	0.19 - 0.51
3.26	22:0 + 20:4	9.8	8.7 - 11.0
3.73	22:1*	0.56	0.28 - 0.76
4.37	23:0	0.49	0.18 - 0.74
5.86	24:0	3.0	2.5 - 4.3
6.53	24:1	3.7	2.2 - 4.5
8.62	22:6*	12.7	10.8 - 13.4

*Tentative identification.

acids, while the lecithin isolated from brain had a high content of palmitic (44%) and oleic (25%) acids. The sphingomyelin-fatty-acid composition was similar to phosphatidyl choline rather than the cerebroside of the brain with palmitic, stearic, and oleic acid accounting for over 60% of the total fatty acids. Fatty aldehydes were found mainly in fractions 5 and 6, and were probably derived from serine and ethanolamine plasmalogens. The saturated aldehydes were largely myristal and palmital, with slight traces of stearal, arachidal, and behenal. Palmitoleic aldehyde was the main unsaturated component, and unsaturated aldehydes with chain lengths longer than 16 carbons were not detected.

Table 5. Percentage hydroxy fatty acids in rat-brain-lipid fractions isolated from silicic-acid column

Fraction	Percent
Whole brain extract	11
1	Not analyzed
2	Not analyzed
3	53
4	91
5	0
6	0
7	20
8	0
9	0
10	0

The sulphatide-fatty-acid composition, fraction 7, was similar to that of the fraction 4 but this fraction was also contaminated by material from the preceding glycerol phosphatide, and hence, the detailed data are not included. Peaks 1 and 2 were not analyzed because of insufficient material, and no fatty-acid analysis was performed on the neutral lipid fraction.

DISCUSSION

The amount of lipid extracted from the whole brain in these experiments is somewhat higher than the values given in the previous literature (20, 21). This may be because of the extraction method used in this study or variation in the lipid composition of species and individual animals. In a previous study of mouse liver (19), this extraction procedure did not increase the total-lipid recovery from that obtained by earlier investigators.

Methanol-chloroform extractions, since being introduced by Folch *et al.* (22), have become the method of choice for most routine total-lipid extractions reported in the literature. Several authors, however, have investigated the method and found it to have certain disadvantages.

Folch *et al.* (23) reported proteo-lipids were found in the organic phase in the extraction of brain and other tissues. Getz and Bartley (24) and de Iongh and van Pelt (25) reported incomplete extraction of liver lipids using methanol-chloroform extraction. In all of these studies the investigators followed the recommended ratio of tissue to solvent reported by Folch *et al.* (22) of 20 to 1 volume to weight of tissue. In tissues extracted in this laboratory (except serum) a ratio of solvent to tissue of about 200 to 1 has been used. This ratio has been

Table 6. Nonhydroxy-fatty-acid and fatty-aldehyde composition in chromatographic fractions isolated from silicic acid

(Mass % methyl esters recovered)

Retention time relative to methyl stearate	Fatty-acid ester or dimethyl acetal (DMA)	Chromatographic fractions analyzed 3	4	5	6	8	9	10
0.15	12:0 DMA*			0.01	0.01	0.02	0.01	
0.17	12:0	0.03	0.34	0.02	0.03	0.05	0.02	0.08
0.21	13:0			0.01		0.04	0.03	0.06
0.25	14:0 DMA*			0.18	0.84	0.03	0.10	
0.30	14:0	0.14	1.0	0.21	0.75	0.06	0.17	0.67
0.41	15:0					0.03	0.08	0.33
0.45	16:0 DMA			0.27	1.3		0.05	
0.55	16:0 + 16:1 DMA	1.8	13.0	1.6	7.0	3.0	43.9	20.4
0.64	16:1	0.46	2.2	0.26	1.2	0.26	0.54	0.73
0.75	17:0	0.11	0.20	0.10	0.13	0.05	0.25	0.24
0.85	18:1 DMA			0.05	0.04			
1.00	18:0	2.5	15.6	38.5	29.0	7.4	13.0	38.9
1.14	18:1	3.4	14.2	12.2	15.5	24.7	25.0	8.8
1.41	18:2 + 19:0	0.68	1.9	0.46	0.27	0.28	0.89	0.26
1.53	20:0 DMA*			0.06	0.08			
1.79	20:0	2.8	5.3	0.57	0.37	1.0	0.39	4.2
2.03	20:1*	0.24	1.8	2.3	2.9	8.3	2.0	0.92
2.38	21:0	0.17		0.27	0.19	1.16	0.20	0.36
2.67	21:1*	0.13		0.15	0.13	0.26		
2.88	22:0 DMA*			0.47	0.41			
3.26	22:0 + 20:4	9.9	10.5	5.2	11.4	20.6	6.4	3.8
3.73	22:1*	2.0	3.2	0.69	0.34	0.93	0.32	1.1
4.37	23:0	3.0	2.3	0.23	0.10	0.23	0.14	0.91
4.88	23:1*	0.84	1.4	0.13	0.06	0.26	0.11	1.5
5.86	24:0	25.8	9.7	5.0	4.7	9.7	1.2	2.9
6.53	24:1	38.8	11.5	2.5	1.4	1.0	0.51	11.9
7.88	25:0	2.2						0.63
8.62	22:6*	2.2	5.8	28.8	21.5	21.6	4.6	1.3
10.37	26:0*	2.8						
Saturated		41.4	47.4	46.1	45.0	21.8	59.5	63.3
Unsaturated		58.0	52.5	53.9	54.7	78.2	40.4	36.7

*Tentative identification.

found to give complete extraction of the lipid in tissue with a single extraction and to extract little non-lipoid substances. For instance, lyophilization of the aqueous phase from brain tissue extracts revealed only 1%-chloroform-soluble substances in the dry residue. However di- and tri-phosphoinositols are not soluble in dry chloroform (Dr. C. E. Ballou, personal communication) but were probably present in this residue. No attempt was made to recover these compounds as they were not eluted from silicic-acid columns under the conditions described here.

It is quite interesting that no proteo-lipid of any kind (Folch and Lees (26) described three types of proteo-lipids in their work which differed in solubility properties and extractability) was found by this extraction method. It would appear that binding of the lipid to protein in these compounds is little if any stronger than that found in other lipoprotein; i.e., they can be disrupted by a simple solvent extraction under mild conditions. It may be that the proteo-lipids are fragments resulting from incomplete extraction of brain lipoproteins, or recombinations of lipids, proteins and water during the washing procedure.

In recent publications, particularly those of Davison and co-workers (2,27), there have been considerable data published on the lipid composition of brain tissue. While almost all of these data were obtained by hydrolytic and chemical means, there is general agreement between the work reported here and other studies (2, 27, 28).

Adams and Davison (29) have reported the total absence of cholesterol esters in adult human brain, and none were detected in the adult rat in this study. The composition of the neutral lipids of brain found in this work is generally similar to that reported in other studies except for the presence of a small unidentified hydrocarbon fraction.

There is still no firm agreement on the relative amounts of certain phospholipids present in brain, although in recent work a more consistent pattern has developed. Particularly striking has been the lack of information on the lysophosphatidyl ethanolamine content of brain phosphoglycerides. In 1956 Debuch (30) obtained lysophosphatidyl ethanolamine from brain but indicated it had been obtained from hydrolysis of ethanolamine acetals. Other workers also reported evidence for lysophosphatidyl ethanolamine, and Long and Staples (31) in 1961 gave the first strong indication that it was a natural component of brain lipids rather than an artifact of the isolation procedure.

Biran and Bartley (3) in a study of rat-brain-lipid composition in whole brain, mitochrondia, and microsomes used silicic-acid-column chromatography to fractionate the total-lipid extracts. However, their column chromatography was carried out using stepwise solvent changes, and fractions containing several

hundred ml of eluate were collected at a time. Thus, while they separated the lipids into neutral lipids, polyglycerol phosphatides, "kephalin", lecithin, and unidentified polar lipids, these fractions were contaminated with hexose-sphingolipids and sphingomyelin and perhaps other unidentified brain lipids, which makes comparison of their data somewhat difficult.

In addition, these authors used indirect methods to analyze the constituents of the various fractions. Thus, they reported 43% inositol and polyglycerol phosphatide in their "kephalin" fraction while only small quantities of these substances were found in this study. Similarly they reported widely varying lysolecithin content among whole brain (negative), mitochondrial, and microsomal fractions, whereas no lysolecithin was detected in this study.

The cerebrosides of brain tissue are usually lumped together as a single class even though they consist of several components (at least three). In this work four distinct peaks are observed. Previously Weiss (5) had also found four hexose-containing peaks in silicic-acid-column chromatography of brain and spinal-cord cerebrosides isolated by solvent fractionation. In recent work by Faillace and Bogoch (4), silicic-acid-column chromatography was used in a multiple fractionation of total-lipid extracts of brain, and a small amount of an amino-glycolipid fraction was eluted by 1:2 chloroform-methanol which may correspond to ganglioside previously isolated by other methods. This substance would probably be lost in the aqueous wash of the lipid extract in the work reported here.

Long and Staples' (1) procedure, also used by Davison and Wajda (2), using alumina columns followed by silicic-acid chromatography or paper chromatography, left the serine phosphatides on the alumina column and caused some hydrolysis of the lipids. In the procedure described here all of the lipids applied to the column are recovered in their natural form as far as could be determined by the analytical techniques employed.

Baker (32) has analyzed the total ester-linked long-chain fatty acids in human nervous tissue and found relatively small but significant differences between the fatty-acid composition of the total brain and its various subfractions. He found in total human brain that palmitic, stearic and oleic were the major fatty acids with very little linoleic present. Baker found only 2.0% unsaturated twenty-two-carbon fatty acids in human brain.

Recently Gertsl et al. (33) analyzed the total fatty-acid composition of human white matter in normal and pathological states. They found more unsaturated fatty acids of the longer chain length than Baker reported, and their data were in closer agreement with what was observed here in the rat. Johnston and Kummerow (34) analyzed total-brain fatty acids in human and chickens and also found results similar to that reported here. These authors reported that most

of the hydroxyl fatty acids were 1-hydroxystearic acid. In both of these reports the amount of linoleic acid detected was quite small. Biran and Bartley (3) analyzed total fatty-acid composition of whole rat brain and also that in the microsomes and mitochondria. Relatively small differences were observed among the three groups and were probably not significant. These authors however did not find as much unsaturated fatty acid as reported in this study.

Fatty-aldehyde composition of whole brain has been studied by Debuch (17) in the rat. In her work, the palmitaldehyde and stearaldehydes were most common, while little myristaldehyde was reported. In this work myristaldehyde was more abundant than stearaldehyde.

Biran and Bartley also reported the fatty-acid composition of their "kephalin", lecithin, and polyglycerol-phosphatide fractions. While their fatty-acid data agree generally with those reported here, there was contamination of these fractions, particularly the "kephalin" with cerebrosides and sulphatides and the lecithin fraction possibly with lysophosphatidyl ethanolamine and sphingomyelin. Nevertheless the same variation of palmitate-stearate ratios between phosphatidyl ethanolamine and lecithin was observed in their study.

The striking differences between the fatty-acid composition of the various glycerol phosphatides found in this study of brain lipids and in other reports of brain and various tissues raises a question about the synthetic mechanisms involved for these compounds. The well-known scheme demonstrated by Kennedy and co-workers (35, 36) does not indicate where a specificity for the fatty-acid composition of the final compound can be introduced, as the same enzyme is apparently active in the transfer of the nitrogenous base to diglyceride receptor for both ethanolamine and choline, and perhaps others (serine, inositol). Similarly the scheme of Bremer *et al.* (37) would necessitate the same fatty-acid composition in both phosphatidyl ethanolamine and lecithin formed by the methylation of ethanolamine to choline in the intact phospholipid molecule.

Studies by Radin *et al.* (22, 38) and Carroll (39) have demonstrated that the total cerebroside-fatty-acid composition is largely long-chain saturated fatty acids of 18, 20, and 24 chain length. Trams *et al.* (40) analyzed ganglioside fatty acids and found almost all of the fatty acids (85%) to be stearic acid; they did not indicate if any hydroxy fatty acid were present. Sulphatides have been reported previously to contain cerebronic, lignoceric and nervonic acid (41).

From the findings of this study it appears feasible to separate total-brain-lipid extracts on silicic-acid columns and obtain excellent recoveries of the lipid classes with little or no decomposition. Heretofore certain lipids, such as sulphatides, could be obtained from brain only by relatively laborious

solvent-extraction techniques (42). While this system does not separate all types of lipid classes (for instance, plasmalogens from their diacyl analogues) it does provide a relatively simple one-step quantitative analysis of the major classes. Further separations can then be made if higher purity is desired.

SUMMARY

Methanol-chloroform extracts of whole rat brain were chromatographed on silicic-acid columns using a concave-gradient-elution procedure. The chromatographic fractions thus obtained were analyzed by various chemical methods, thin-layer chromatography and infrared spectrophotometry. Cerebroside, serine and ethanolamine phosphatides, sulphatide, lysophosphatidyl ethanolamine, lecithin, and sphingomyelin were eluted as separate fractions. The fatty-acid and fatty-aldehyde composition of the chromatographic fractions and whole brain was determined. Hydroxy acids were found only in the hexose sphingolipids. The highest percentage of unsaturated fatty acids was found in the amino-glycerol phospholipids. Only small amounts of linoleic acid were detected in whole brain or any fraction.

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Simultaneous Determination of Cholesteryl Esters and Triglycerides in Serum Lipids by Infrared Spectrophotometry

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Cholesteryl esters and triglycerides are two of the principal lipid components in serum. Their relation to lipoprotein structure, lipid transport and metabolism, and to atherosclerosis is under continuing investigation in many laboratories. From present knowledge of the chemical composition of the serum lipoproteins (1), the two major low-density classes are distinguished by their relative contents of cholesteryl esters and triglycerides. Lipoproteins of class S_f^o 0-20 have cholesteryl esters as the predominant lipid constituent, whereas lipoproteins of lower density ($S_f^o > 20$) are characterized by high triglyceride content. Measurements of these lipid components can therefore be correlated with the distribution of lipoproteins in the low-density classes.

A considerable variety of methods exists for the determination of serum cholesterol, and as a corollary, cholesteryl esters. The estimation of triglycerides has in the past been a more formidable problem, and results have been less reliable. The procedure described in this paper is based on high-resolution infrared spectrophotometry. It provides a relatively simple and accurate analysis for cholesteryl esters and triglycerides simultaneously.

Infrared spectrophotometric methods for the analysis of blood lipids have been developed in this laboratory and described in previous publications. In one type of analysis (2) infrared-absorption measurements are made directly on the total lipid extract from serum. Results are thereby obtained in terms of total lipids, total esterified fatty acids, total phospholipids and total cholesterol. From these data it is then necessary to derive values for cholesteryl esters and triglycerides by the usual calculations and assumptions that are made in corresponding chemical analyses. This method has the virtue of great operational simplicity, but it has inherent weaknesses making it inadequate for certain purposes. In its present state of development, it does not provide a good quantitative measurement of triglycerides.

An alternative but more laborious procedure is fractionation of the total lipid mixture by elution from a silicic-acid column, and analysis of the fractions by infrared (3). Cholesteryl esters are isolated and measured as a

separate class, and triglycerides are readily determined as the major component of a second fraction. Phospholipids may be eluted together and measured collectively or subfractionated by a more extensive elution scheme if it is desired to study the various phospholipid components (4). While this combined column-infrared method satisfies the need for direct measurements of cholesteryl esters and triglycerides, it does not lend itself well to large numbers of samples.

The objections to the foregoing procedures can be largely overcome if the analysis is restricted to two components. Phospholipids, therefore, are first removed from the total lipid mixture by a simple batch adsorption on silicic acid as suggested by Van Handel (5). Cholesteryl esters and triglycerides are the major constituents in the non-adsorbed lipid, and the infrared measurements are made on this mixture.

The simultaneous analysis of the two components is based on the fact that the ester-carbonyl-absorption frequency (maximum) for cholesteryl esters is different from that of triglycerides by about 14 cm^{-1}. This difference is readily observable with the resolving power of a sodium-chloride prism. However, in order to make quantitative measurements of both components in a mixture, it is necessary to have higher resolution and also the capability of a precise, reproducible setting of the frequency (or wavelength). The analysis has been developed using a Perkin-Elmer Model 421 grating spectrophotometer. However, it is presumed that comparable grating instruments of other manufacturers would be equally satisfactory. Limited data have indicated that the resolving power of a calcium-fluoride prism is also sufficient for this purpose.

Fig. 1 illustrates this pair of absorption bands with a five-fold abscissa expansion from the normal scale. The degree of overlapping is apparent.

MATERIALS AND METHODS

Solvents used are reagent grade. Errors attributable to solvent blanks are negligible at this level of sample size. Evaporations are carried out on a hot plate at about 50°C, using a current of nitrogen.

EXTRACTION OF LIPIDS. Extraction is carried out on 1 ml aliquots of serum, using 2:1 chloroform-methanol and the method of Sperry and Brand (6). Other methods may be suitable, provided they yield complete extraction of the neutral lipids. Failure to extract phospholipids completely is of no consequence. The lipid extract is taken to dryness and redissolved in 0.5 ml of chloroform.

ADSORPTION OF PHOSPHOLIPIDS. Approximately 50 mg of silicic acid (Bio-Rad Laboratories, Richmond, Calif.) is placed in a 2-dram (8 ml) screw-cap glass vial that has been calibrated and scribed at a volume of 6.0 ml. (The screw cap

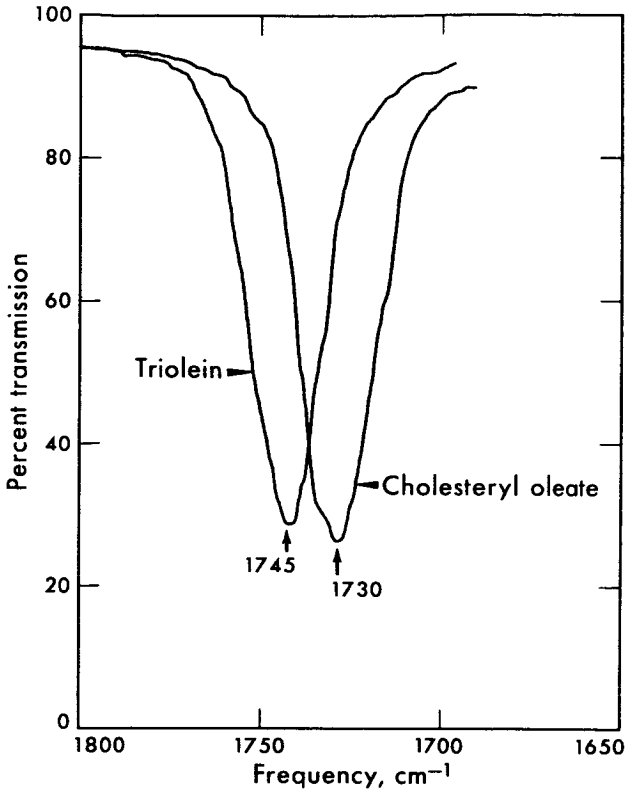

Figure 1. Carbonyl absorption bands of cholesteryl oleate and triolein, showing extent of overlapping. Frequency scale expanded to five times normal.

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should be lined with polytetrafluoroethylene to prevent contamination by plasticizer from the ordinary liner). 0.5 ml of chloroform is added to wet the silicic acid. A solution of the lipid sample in 0.5 ml of chloroform is added and mixed with silicic acid in suspension. The vial is then filled to the 6-ml mark with acetone and tightly capped. By inversion and rotation the contents are thoroughly mixed. The mixing is repeated once or twice during a period of 15-20 min, after which the silicic acid is allowed to settle for at least 30 min. Five ml of the clear supernatant are then transferred to a clean dry vial and evaporated to dryness.

INFRARED MEASUREMENTS. The dried aliquot of non-adsorbed lipids is dissolved in an accurately measured volume of carbon tetrachloride to give a total concentration of 3-5 mg/ml. The volume required is usually between 0.6 ml and 1.2 ml. If no prior information is available concerning the amount of lipid, a trial may be made at a volume of 0.7 ml. If the measured absorbances are not within the range 0.2-0.6, it may be desirable or necessary to repeat the measurements at a different concentration.

The absorption cell has a nominal thickness of 1.0 mm. A similar cell containing pure carbon tetrachloride is used in the reference beam of the spectrophotometer. Absorbances are measured at 1745 cm^{-1} and 1730 cm^{-1}, with a slit

width of 0.25 mm. This corresponds to a 2 x 1000 slit program setting on the instrument controls.

Since the two frequencies are quite close together, and neither is ordinarily on the (resultant) band peak, it has been considered preferable to set them manually and record the absorbances at stationary scale positions rather than to scan the band and make readings from the recorded curve. Scale settings can be made manually to within 0.2 cm^{-1}.

With the equations given in the next section, the measured absorbances are used to calculate concentrations in the CCl_4 solution. They are multiplied by the volume of carbon tetrachloride to give weights (in mg, if mg/ml is the concentration unit). Further conversion to mg per ml of serum is made by the factor 6/5, which accounts for the volume change in the adsorption step. If an aliquot was taken in the extraction step, an additional factor is necessary.

CALIBRATION. With pure triolein (Hormel Foundation) and pure cholesteryl oleate (Applied Science Labs) as standards, absorption coefficients were obtained for triglycerides and cholesteryl esters at the two selected frequencies. There are slight variations in absorptivity with concentration, but these have been neglected on the premise that very high and very low concentrations will be avoided. The values used for calculation correspond to the expected concentration range of total ester carbonyl.

According to the standard mathematics for this type of analysis (7), the measured absorbance is the sum of contributions from two components. Putting in the selected absorption coefficients,

$$A^{1745} = 0.165\ C_{TG} + 0.019\ C_{CE}$$

$$A^{1730} = 0.040\ C_{TG} + 0.080\ C_{CE}$$

Where A = absorbance
C = concentration
TG = Triglyceride
CE = Cholesteryl ester

Solving to obtain expressions for concentrations in terms of absorbances,

$$C_{TG} = 6.44\ A^{1745} - 1.53\ A^{1730}$$

$$C_{CE} = -3.22\ A^{1745} + 13.27\ A^{1730}$$

This pair of equations is applied in the analysis of samples, using absorbances measured as described above.

RESULTS

The two-component infrared analysis was tested, using known mixtures of

Table 1. Analyses of known mixtures of cholesteryl oleate and triolein

Sample	Component	Wt in mg		% Error
		Known	Found	
1	CE	3.91	4.08	+4.4
	TG	2.34	2.26	-3.4
2	CE	8.16	8.24	+1.0
	TG	2.67	2.70	+1.1
3	CE	6.63	6.95	+4.8
	TG	7.24	7.45	+2.9
4	CE	4.18	4.40	+5.3
	TG	14.86	15.0	+0.9
5	CE	1.06	1.20	+7.6
	TG	1.98	2.02	+2.0
6	CE	4.54	4.52	-0.4
	TG	2.70	2.65	-1.9

triolein and cholesteryl oleate. Results are given in Table 1.

As an evaluation of the overall method, some analyses were carried out on replicate samples of serum starting with extraction and proceeding through the adsorption step to the infrared measurements. The results were compared with cholesteryl ester and triglyceride values for the same serum obtained by silicic-acid chromatography and infrared analysis. These data are given in Table 2.

The results given in Table 2 do not take into account the variability in the amount of lipid extracted. If the figures given are converted to percent of total lipid, the agreement is generally improved.

DISCUSSION

This analysis is based on the primary assumption that cholesteryl esters and triglycerides are the only significant absorbers at the frequencies selected for infrared measurement. In order to meet this requirement, the following are necessary:

1. The silicic acid adsorption must provide a clean separation, taking out all of the phospholipids and none of the components to be measured.
2. The contribution of other non-absorbed lipids must be small enough to be negligible.

In the years since it was reported by Borgstrom (8) that phospholipids could be separated from neutral lipids by chromatography on silicic acid, the accumulated experience of many workers has left little doubt as to the efficacy of this separation when carried out on columns. The use of silicic acid in batch adsorption

Table 2. Overall reproducibility and comparison with results obtained by column chromatography (mg per ml serum)

Sample	Component	Two-component infrared	Chromatography infrared
1	CE	2.93, 2.67, 2.72, 2.68	2.58, 2.64
	TG	1.66, 1.58, 1.61, 1.58	1.69, 1.71
2	CE	2.80, 2.80, 2.58	2.72, 2.61
	TG	0.75, 0.78, 0.71	0.78, 0.77
3	CE	3.12, 3.30, 3.39, 3.25	3.01, 2.86
	TG	5.20, 5.12, 5.24, 5.19	5.03, 4.70

has been mentioned by Van Handel (5) and by Mendelsohn and Antonis (9). In our experiments we have varied the amount of silicic acid over the range from 30 to 80 mg with no significant difference in results. Use of either acetone or chloroform also gave equivalent answers. Phosphorus analyses were not done on the non-adsorbed lipids, but no evidence of phospholipid was detectable in their infrared spectra. It seems probable that any of several combinations of adsorbent (silicic acid, Florisil, Zeolite) and solvent (chloroform, acetone, ether) would be satisfactory. Direct extraction from serum by the procedure of Van Handel and Zilversmit (10), or of Mendelsohn and Antonis (9), would simplify the analysis still further.

The non-adsorbed lipid fraction contains unesterified cholesterol and unesterified fatty acids in addition to the principal components. The cholesterol molecule has no carbonyl group, and its contribution to the measured absorbances--for amounts expected in serum--is less than the error of measurement. The absorption maximum of free fatty acids is displaced from those of the ester carbonyls, but there is a slight overlap with those bands. From calibration data it is estimated that error resulting from the presence of fatty acids is ordinarily less than 5%. Further investigation of this point is needed, and it appears feasible to make a correction for free fatty acids if necessary.

If acetone is not completely removed from the lipid on evaporation, its carbonyl absorption would presumably contribute an error. As a precaution it is advisable to redissolve the lipid mixture in carbon tetrachloride and re-evaporate.

The possibility that lipid oxidation products containing carbonyl groups could be a source of error needs to be considered. It has been assumed that this is not a serious concern for fresh lipid samples, handled with some care. Data that are being accumulated on stored serum samples may help to clarify this point.

SUMMARY

With the use of a high-resolution grating infrared spectrophotometer, it is possible to analyze mixtures of cholesteryl esters and triglycerides on the basis of the difference in their ester carbonyl absorption frequencies. In the analysis of serum lipids, the preliminary removal of phospholipids is accomplished by a simple batch adsorption on silicic acid. Estimated errors are less than 5%.

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Analysis of Low-Density Lipoproteins by Preparative Ultracentrifugation and Refractometry

Frank T. Lindgren, Alexander V. Nichols, Robert D. Wills,
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The analysis of blood lipids is an important factor in the treatment and prognosis of disease states whose principal biochemical manifestations are an elevation of particular blood lipids and lipoproteins. Because of the principal involvement of both glyceride and cholesterol in the lipid elevations associated with such a disease as coronary arteriosclerosis and its consequences, a glyceride and cholesterol measurement would provide important information to the clinician who must treat the patient. Usually, a glyceride determination can be made by either a glycerol analysis (1) or by means of an infrared analysis (2). A total gravimetric lipid measurement, although tedious and not routinely compatible with the usual clinical laboratory procedures, can also, in combination with a serum cholesterol measurement, provide a good estimate of the $S_f20\text{-}10^5$ lipoproteins (3) and, hence, of serum glyceride. In any event, it is not easy to obtain simultaneously a reliable serum cholesterol and serum glyceride determination in the clinical laboratory.

A more direct picture of the native state of the blood lipids associated with coronary arteriosclerosis is provided by an ultracentrifugal analysis of the low-density-lipoprotein spectra. Much of the available data (4,5) on these low-density lipoproteins have been given for four lipoprotein groups; the $S_f0\text{-}12$, $S_f12\text{-}20$, $S_f20\text{-}100$ and $S_f100\text{-}400$. However, from the point of view of important metabolic responses and particularly from considerations for treatment (dietary or pharmacological), these four lipoprotein classes may be appropriately grouped into the $S_f0\text{-}20$ and $S_f20\text{-}400$. On the basis of hydrated density, these lipoproteins may be described as low density (LD) (1.006-1.050 g/ml) and very low density (VLD) (0.92-1.006 g/ml) lipoproteins, respectively. Biochemically, the former correspond to the principal cholesterol-bearing lipoproteins and the latter to the major glyceride-bearing lipoproteins. Unfortunately, the complete ultracentrifugal determination of these lipoproteins, although providing intrinsically greater information than the method here presented, is both expensive and technically difficult. The greater portion of the difficulty is in the final analytical ultracentrifugal analysis, not in the preparative stage of lipoprotein isolation, a comparatively simple procedure. The following method, a refinement of a previous procedure (6), avoids the analytical difficulties by employing a relatively simple, accurate and reproducible refractometric analysis of the low-density-lipoprotein fractions obtained by preparative ultracentrifugation.

MATERIALS AND METHODS

All preparative runs were made at 18°C in a Spinco Model LH ultracentrifuge equipped with a "vacuum sentinel" (7) (also p. 107) to minimize sample loss in case of vacuum failure. This seemed desirable because for this procedure 9 ml of serum are required for optimum analysis. Thus in most cases insufficient serum might be available for a duplicate analysis. Isolation of lipoproteins was accomplished after 18 hr of centrifugation at 40,000 rpm, in a 40.3 rotor.

For each lipoprotein-containing sample two fractions were pipetted in depth, the top milliliter containing quantitatively the lipoprotein fraction and the second milliliter providing reference salt background for that sample. Pipetting was done in a darkened room on a fixture equipped with a focused light beam allowing visualization of the lipoproteins by their tyndall scattering. Fractions collected in 1-ml volumetric vials were transferred for storage into 9-ml air-tight screw-cap vials fitted with teflon gaskets. Refractive index measurements were made with a Bausch and Lomb precision Abbe' refractometer (8), with a range of n_D from 1.203 to 1.508, thermostated to 26.00°C ± .02°C with a temperature controller (Precision Scientific Co.).

In essence, this method consists of isolating by preparative ultracentrifugation two lipoprotein fractions from each serum sample. The first fraction is the glyceride-rich low-density lipoproteins (VLD) less dense than 1.006 g/ml (the $S_f20\text{-}10^5$). (Unless otherwise stated, all densities are given at 20°C.) The VLD lipoproteins are obtained ultracentrifugally without prior density manipulation of serum. For this purpose 6 ml of serum are directly centrifuged for 18 hr at 40,000 rpm. With regard to density considerations and manipulations, serum may be considered to be 94% by volume a salt solution (primarily NaCl) of density 1.0065 g/ml. If 6 ml of serum are unavailable for the VDL fractionation, the difference between the available serum and the recommended 6-ml capacity of the preparative tube is substituted with an appropriate volume of a 0.202 Molal NaCl solution (ρ_{20} = 1.0065 g/ml). Solutions are given in molal concentrations (moles/1000 g H_2O) so as to avoid temperature dependence.

The total low-density-lipoprotein fraction ($S_f0\text{-}10^5$) or TLD fraction is obtained by centrifuging a solution consisting of 3 ml serum and 3 ml of a 3.278 Molal NaCl salt solution (ρ_{20} = 1.1168 g/ml). The background salt solution resulting from the use of the above volumes serves as the reference background to which all TLD runs must be brought. Thus, if only 2 ml of serum are available, a salt solution equivalent in density to the reference background is obtained by first adding 0.94 ml of a 0.202 Molal NaCl solution to the 2 ml of serum. Then, by adding 3 ml of the 3.278 Molal NaCl salt solution to this mixture, the final background salt solution will be the same as that resulting from mixing 3 ml serum and 3 ml 3.278 Molal NaCl. It should be pointed out that this density,

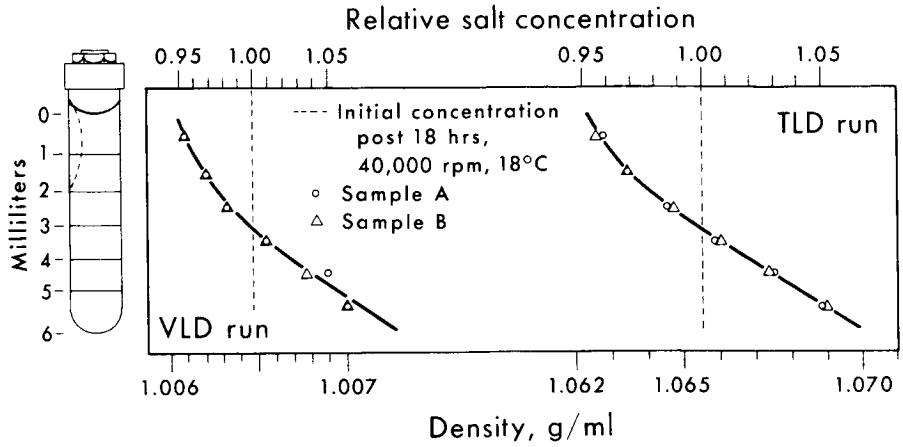

Figure 1. The ultracentrifugal redistribution of NaCl in both the very-low-density (VLD) and total-low-density (TLD) preparative runs.

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1.0652 g/ml, is the solution density before preparative ultracentrifugation and is equal to 1.0630 g/ml at 26°. However, during preparative centrifugation, appreciable salt redistribution occurs from the top to the bottom of the preparative tube. The extent of redistribution is dependent on the nature and concentration of the salt as well as on the time and conditions of ultracentrifugation. Under conditions of this method, redistribution of salt for both VLD and TLD runs are shown in Fig. 1. Thus, after conditions of centrifugation presented here, the density of the top 1 ml of the VLD run falls from approximately 1.0065 g/ml to 1.0061 g/ml and the top 1 ml of the TLD run is reduced from approximately 1.0652 g/ml to 1.0622 g/ml. This salt redistribution must be taken into account fully if we are to measure lipoprotein concentrations accurately.

The actual calculation of lipoprotein concentrations were made using the following relation:

$$\text{lipoprotein concentration, mg \%} = (\Delta S_{TF} - \Delta S_B)\frac{(K_1)}{(C_o)}\frac{(1.000)}{(S.R.I.)}$$

where: S_{TF} = Abbe' scale reading increment of top fraction above the water reference.*

S_B = Calculated Abbe' scale-reading increment of top-fraction salt background above the water reference.

K_1 = Instrument-dependent conversion factor of scale-reading increment to refractive-index increment, our values:

K_1 (TLD) = 5.30×10^{-3}

K_1 (VLD) = 5.48×10^{-3}

*A water reference reading is essential before and after each set of refractometric readings, and it is further recommended that additional water readings be taken after every tenth sample reading.

C_o = Concentration factor of lipoprotein factor relative to initial serum.

S.R.I. = Specific refractive increment; 0.00171 for VLD and 0.00154 for TLD runs. As used here S.R.I. is the increase in refractive index of the indicated salt solutions resulting from the presence of 1 g of lipoprotein per 100 ml solution, measured at 26°C.

The centrifuged background salt solution present in the centrifuged top fraction is evaluated from the second ml fractions from both the VLD and TLD runs. Refractometry on these fractions allows accurate calculations of the background refractive index of the top ml aliquots from each individual VLD and TLD run.

Experimentally, the ratio of the refractive-index increment, above the water reference, of the first ml to the second ml, V_1^0/V_2^1 of both the TLD and VLD salt-reference runs as described here, is 0.985. Thus, multiplying the refractive-index increment of the respective 2nd-ml reference fractions by 0.985 yields, in each case, the refractive increment of the actual salt background of the lipoprotein-containing fraction. Further, if pipetting difficulties occur, a pooled 1st- and 2nd-milliliter lipoprotein fraction can be taken, together with a 3rd-milliliter reference fraction. In this case, the ratio of V_2^0/V_3^2 for the salt-background refractive increment is approximately 0.975 for both the TLD and VLD runs. In all cases, the concentration of lipoproteins is obtained from the difference in refractive-index increment (above the water reference) of the lipoprotein-containing fractions and the calculated refractive increment for its respective salt background.

Probably the most convenient and accurate refractometric analysis of small quantities of solution can be made with a precision Abbe' refractometer, temperature controlled to at least ± 0.1°C. The readings should be made at a constant temperature somewhat above that of room temperature— in this procedure at 26°C. In this method readings should be made in a manner so as to take full advantage of the accuracy of the precision Abbe', with scale readings estimated to the nearest ± 0.005 units. Over the range of measurements, relative accuracy of refractive-index measurements* can be made to within approximately ±0.00003 refractive-index units. An ordinary Abbe' refractometer cannot give sufficient accuracy to be successfully used for this method. A Pulfrich refractometer (9) can provide somewhat greater accuracy of measurement than the precision Abbe' but because of the convenience and adequate accuracy of the precision Abbe', the latter is recommended.

*A photoelectric sensing device can be used more effectively to set, with a servo-mechanism, the quadrant of the refractometer to the critical angle characteristic of each sample. Further, with this provision a direct scale readout is possible.

Refractometry can be done with only one drop of the lipoprotein fraction by taking the "reflection" reading. Thus, nearly all the fraction may be available, if desired, for other lipid analyses. The drop of lipoprotein solution should be placed slightly above the center of the outer ground-glass prism and the prism closed immediately. Thereafter, a time delay of exactly one minute between application of the sample and taking the refractometric reading is recommended. This is sufficient for approximate temperature equilibration but insufficient for any significant evaporation. After each reading the surface of the opposing prisms is thoroughly washed by directing against each prism a stream of about 50 cc of distilled H_2O from a polyethylene washing bottle. Thereafter, the prism surfaces are wiped (unidirectionally) with a non-abrasive wiper (Kimberly Clark Type 900-S). To insure a dry prism surface, an unheated air stream is directed for 5 sec onto each prism surface from a hair dryer (Oster, Model 202).

For the purposes of converting refractive-index measurements to lipoprotein concentrations, a specific refractive increment of 0.00171 units (10) is used here for the VLD (S_f20-10^5) fraction and a specific refractive increment of 0.00154 units (11) is used for the TLD (S_f0-10^5) fraction. Calculation of the cholesterol-rich S_f0-20 lipoproteins is obtained by difference. A refractometric Atherogenic Index ($A.I._R$) approximately equivalent to the analytic ultracentrifugal A.I. (12) value may be obtained by the following relation:

$$A.I._{U.C.} \simeq A.I._R = 0.100\ (S_f0\text{-}20, \text{ concentration in mg/100 ml}) + 0.199\ (S_f20\text{-}10^5, \text{ concentration in mg/100 ml}).$$

RESULTS

Table 1 shows a comparison of 30 serum analyses by analytic ultracentrifugation and refractometric determinations. The mean total low-density values (TLD) obtained by both methods agree very closely, which suggests that there are minimal optical dispersion effects and minor differences between the methods. Furthermore, in the serums studied the results suggest that there existed relatively low concentration of S_f400-10^5 lipoproteins. The elevated S_f20-10^5 lipoprotein values, determined by refractometry over the S_f20-400 values determined by analytic ultracentrifugation, suggest that the actual fractionation of the VLD run (S_f20-10^5) probably obtains some lipoproteins of flotation rates lower than S_f20. An estimate of the actual flotation rate of a lipoprotein of 1.006 g/ml hydrated density is approximately S_f16 (13). Further, the values of the S_f0-20 and S_f20-400 reported here have not been corrected for S_f versus c and Johnson-Ogston effects (14,15). These corrections though relatively small for the two major lipoprotein groups (5) would nonetheless tend to increase the value of S_f20-400 and decrease the value of the S_f0-20 lipoproteins. However, the primary effect under consideration is that of the self slowing (S_f versus c effect) of the lipoproteins upon each other. For the mean S_f0-20 concentrations studied here, the average S_f20 lipoprotein, present in a medium of about 1.4% S_f0-20 lipoproteins, would exhibit an actual flotation rate of about S_f17.

Table 1. Comparison of analytic ultracentrifugal data with refractometric lipoprotein determinations

Serum No.	Conc (TLD)	S_f0-20 (U.C.)	S_f0-20 (Δn)	S_f20-400 (U.C.)	Conc (VLD)	S_f20-10^5 (Δn)	S_f0-400 (U.C.)	S_f0-10^5 (Δn)
1	3 Co	407	380	11	6 Co	59	418	439
2	3	431	365	73	6	109	504	474
3	3	405	372	39	6	56	444	428
4	3	448	443	95	6	174	543	617
5	3	505	487	95	6	182	600	669
6	3	456	416	90	6	155	546	571
7	3	482	435	101	6	171	583	606
8	3	356	352	53	6	64	409	416
9	3	283	245	21	6	45	304	290
10	3	340	356	18	6	37	358	393
11	3	388	349	6	6	27	394	376
12	3	365	344	38	6	72	403	416
13	3	432	375	54	6	59	486	434
14	3	352	311	63	6	88	415	399
15	3	582	558	61	6	93	643	651
16	3	268	271	2	6	13	270	284
17	3	344	282	48	6	37	392	319
18	2 Co	347	296	627	6	742	974	1038
19	3	320	315	12	6	38	332	353
20	3	571	535	141	6	139	712	674
21	3	277	259	5	6	8	282	267
22	3	329	328	2	6	8	331	336
23	3	498	494	109	6	152	607	646
24	3	269	247	203	6	238	472	485
25	3	447	382	216	6	264	663	646
26	3	188	181	238	6	270	426	451
27	2 Co	579	474	99	6	104	678	578
28	1.5 Co	1283	1118	756	6	733	2039	1851
29	3	225	240	82	6	131	307	371
30	3	499	427	117	6	200	616	627
Mean values (mg%)		423	388	116		149	538	537

Table 2 compares the results of seven VLD runs analyzed by both analytic ultracentrifugation and refractometry. For such analyses, a specific refractive increment (S.R.I.) of 0.00171 is used for refractometry and a S.R.I. of 0.00154 employed for analytic ultracentrifugation. For the latter analysis, solid NaCl was added in the amount of 87.2 mg for each milliliter of VLD fraction. This amount, assuming the lipoprotein fraction consists of 1% by volume lipoprotein, is sufficient to bring the small-molecule background density to 1.063 g/ml. Of course, introducing this amount of salt into each VLD fraction increases the volume and, hence, dilutes the original VLD fraction by a factor of approximately 0.973. Taking this factor into account, the actual results obtained by each method compare very favorably with one another suggesting again that optical-dispersion differences and significant concentration of S_f400-10^5 lipoproteins are apparently not a problem in evaluating the VLD lipoproteins by refractometry.

A measure of the reproducibility of this method, particularly when

Table 2. Comparison of analytic ultracentrifugal data with refractometric data

Serum No.	Conc	$S_f20-400$ (UC)	S_f20-10^5 (Δn)
23	6 Co	150	152
24	6 Co	211	238
25	6 Co	260	264
26	6 Co	258	270
27	3 Co	105	104
28	6 Co	836	733
30	6 Co	176	200
	-	285 (mean)	280 (mean)

different amounts of serum are used, is shown in Table 3. Here, for a single serum sample, a reproducibility of approximately ± 5% is maintained for both the VLD and TLD runs. An additional measure of anticipated error may be calculated from duplicate analysis to give a standard error of estimate. For six duplicate analyses of the VLD and nine duplicate analyses of the TLD runs a σ_{SEE} = 5 mg% and a σ_{SEE} = 27 mg% was obtained for the S_f20-10^5 and S_f0-10^5 lipoproteins, respectively. This accuracy and reproducibility compares favorably with that observed for lipoproteins determined by analytic ultracentrifugal analysis (5).

DISCUSSION

It is evident that the refractometric determination of the low-density lipoproteins is not equivalent to an analytical ultracentrifugal determination. In the first place, lipoproteins above S_f400, although usually present at relatively low abundance, are measured by refractometry but are not customarily measured by analytic ultracentrifugation. On the other hand, the more subtle features of the entire low-density-lipoprotein distribution, revealed in detail with an analytic ultracentrifuge determination, are unavailable with refractometry. Further, certain discrepancies still exist in the determination of the low-density lipoproteins by refractometry and by analytic ultracentrifugation. Such small discrepancies may be due in part to the presence of non-migrating lipoproteins in each of the lipoprotein fractions, thus giving an apparently lower concentration of lipoproteins detected by the schlieren optical system. Further, some small dispersion effects may be anticipated in that the refractive index measurements with the precision Abbe' are made with the Na D_{12} lines (5890Å and 5896Å) whereas the analytic schlieren diagram giving dn/dx (and by integration a total lipoprotein Δn) is obtained primarily with the green line of the Hg arc (5461Å). Despite these small discrepancies the principal low-density lipoproteins may be measured refractometrically and compared to analytic-ultracentrifuge values even in the absence of a full theoretical explanation of these differences.

Table 3. TLD- and VLD-lipoprotein reproducibility on a single serum sample (refractometry)

Lipoprotein fraction	$S_f 0\text{-}10^5$	Lipoprotein fraction	$S_f 20\text{-}10^5$
TLD 3 Co	945	VLD 6 Co	489
TLD 3	928	VLD 6 Co	473
TLD 3	934	VLD 5 Co	471
TLD 2	917	VLD 4 Co	457
TLD 1	888	VLD 3 Co	439
		VLD 2 Co	450

SUMMARY

A simplified method for the analysis of both the glyceride-rich $S_f 20\text{-}10^5$ and the cholesterol-rich $S_f 0\text{-}20$ low-density lipoproteins is presented. It consists of serum-lipoprotein fractionation by preparative ultracentrifugation and subsequent quantitative analysis by refractometry. Comparison of this technique with the technically more difficult analytic-ultracentrifugal methodology reveals comparable results for these two principle low-density-lipoprotein groups. One of the advantages of this procedure is that it provides a reliable and reproducible means for quantitating the principal glyceride-bearing-lipoprotein group--the $S_f 20\text{-}10^5$ lipoproteins.

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'Vacuum Sentinels' for Preparative and Analytical Ultracentrifuges

Frank T. Lindgren and Frank T. Upham

Vacuum failure in the preparative ultracentrifuge and cell leakage in analytical ultracentrifuges are occasional instrumentation hazards for those engaged in ultracentrifugal studies. These failures can involve loss or damage of critical biological material and may result in breakage of analytical cells. In many instances, the serum or other biological specimens lost may be a critical part of a very important research effort.

During preparative ultracentrifugation, a vacuum loss usually results from either failure in the pumping system or from deterioration in the drive seal. Occasionally, this latter failure suddenly occurs toward the end of the useful life of the drive mechanism. Although persistent loss of drive oil will ultimately trip the oil-reservoir switch, and thus safely stop the ultracentrifuge, this may not happen before the rotor and its contents undergo damaging heating by the increased air friction without the investigator being aware of it.

Vacuum losses can be readily avoided by the installation of a "vacuum sentinel" on the preparative ultracentrifuge, which will automatically shut off the drive mechanism instantly should the vacuum fall below a pre-set level, e.g. 5×10^{-3} mm Hg pressure (5 μ). Thus, in the event of vacuum failure, the samples are neither damaged nor lost but may be re-processed after the centrifuge is repaired or be re-run in another centrifuge.

A second potential application for a vacuum sentinel is in preparative work where swinging-bucket rotors are employed. During long unattended runs, slow but persistent leakage from one swinging bucket might lead to critical imbalance of the rotor. This could result in destruction of the rotor as well as extensive damage to the ultracentrifuge. Regardless of the consequences, in the event of leakage it would be desirable to stop the centrifuge and allow appropriate repairs to be made before continuing the experiment.

Cell leakage, with attendant vacuum loss, is also an occasional problem in analytical ultracentrifugation. In the event of leakage at relatively high rotor speeds, destruction of expensive cell parts such as quartz windows and centerpieces usually occurs. This is of particular concern where double-sectored

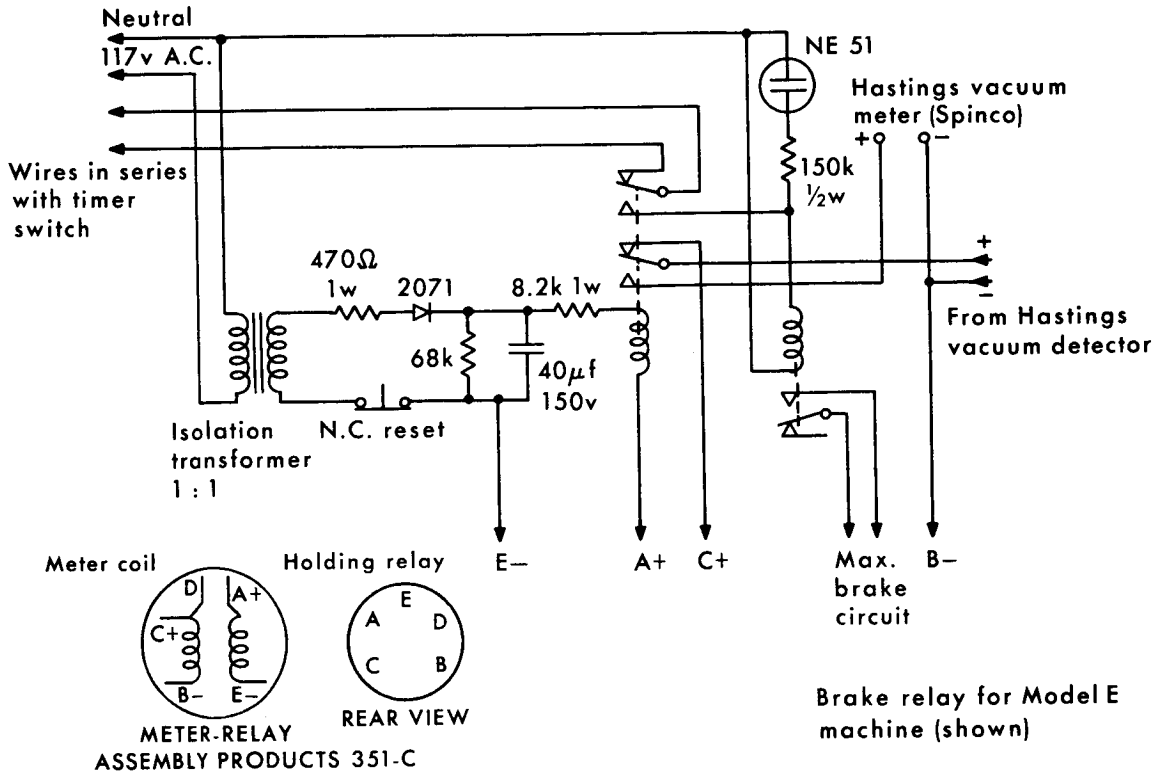

Figure 1. Vacuum sentinel circuit for Spinco Model E or L ultracentrifuge.
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epoxy centerpieces are employed. Therefore, when such cell leaks and subsequent vacuum losses occur, either during acceleration or after the ultracentrifuge has reached full speed, it is advantageous to shut off immediately the drive mechanism and to apply the full brake. In many cases where leakage is slow, even at high operating speeds (52,640 rpm), the vacuum sentinel can automatically shut off and thus sufficiently reduce rotor speed so that the cell itself is undamaged. Further, in unattended runs, there is an advantage in knowing whether or not the centrifugal run has proceeded to completion without perceptible cell leakage. Occasionally, even at full speed, a cell may leak very slightly and yet may subsequently recover. Such an unsatisfactory run might be discovered only after careful analysis of the centrifuge film or plate, at which time rerunning of the sample may not be possible. Thus, the use of a vacuum sentinel provides the capability of decreasing analytical-cell leakage as well as providing automatic monitoring throughout the duration of the analytical run.

COMPONENTS AND INSTALLATION

A vacuum-sentinel circuit, appropriate for either the Spinco Model E or L ultracentrifuge, is shown in Fig. 1. It incorporates an Assembly Products Instrument (API)* 351-C type meter. This meter is not continuous-reading, and

*Assembly Products, Inc., 75 Wilson Mills Rd., Chesterland, Ohio.

therefore we use the original vacuum-gauge meter during the initial pump-down period. When the vacuum-chamber pressure is below a predetermined level, serving as a high pressure limit, the reset button is pressed. This switches the meter circuit from the original Spinco meter to the API meter, releasing the brake circuit, if used, and energizing the normal control system allowing the centrifuge to be started in the usual manner. Should the pressure rise above the preset value, the relay will be tripped and the centrifuge automatically shut off. In addition, this relay circuit may be used to apply the full brake, sound an alarm, or to operate any desired auxillary circuit.

If one is willing to accept a wider "dead zone" during vacuum loss, an API continuous-reading relay-meter can be used along with the manufacturer's recommended control circuit. This meter can be installed in place of the original Spinco vacuum gauge.

The total cost of the parts needed to construct a vacuum sentinel, as described above, is approximately $50.00.

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A Quantitative Determination of the Osmium-Tetroxide-Lipoprotein Interaction

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The investigation of the fine structure of biological material with the electron microscope has depended to a very large extent on the deposition of osmium compounds within the tissue during treatment with a buffered osmium-tetroxide solution. The value of such treatment in the fixing of structure and enhancement of contrast is well accepted. Since electron-microscopic observations have depended so heavily on this material, it is of importance to know the physico-chemical parameters that are concerned in the interaction between osmium tetroxide and the tissue components. Successful interpretation of the electron micrographs is only possible through knowledge of these parameters.

Several studies have been made of the qualitative reaction of osmium tetroxide with biological materials. Altmann in 1894 (1) showed that osmium tetroxide would blacken oleic acid and olein but that the saturated fatty acids, palmitic and stearic acid, and their triglycerides were not affected. Bahr in 1954 (2) extended the observations of blackening by osmium tetroxide to some 250 biologically-important compounds and showed that osmium tetroxide would react with unsaturated lipids and proteins but not with saturated hydrocarbons, carbohydrates, or nucleic acids.

The importance of the unsaturated fatty acids in binding osmium was further emphasized by Wigglesworth (3), in 1957, who felt that osmium-tetroxide staining presented a histology based on lipids as compared to normal protein and nucleic-acid histology. He also presented a hypothesis that there was a polymerization of the unsaturated lipids by osmium cross linking of ethylenic double bonds. This insoluble complex of lipid and osmium is particularly liable to occur in layers of oriented lipid. Again in 1959 Hess pointed out the essentially lipid nature of osmium-binding sites (4).

MATERIALS AND METHODS

Very little information exists as to the amount of osmium bound by biological compounds, and this study was undertaken to provide some data on this point. The material used for interaction with osmium tetroxide was isolated classes of serum lipoproteins. The chemistry of these lipoproteins is well known in terms of amount of protein and lipid, biochemical nature of lipid

Table 1. Osmium uptake by serum lipoproteins

Lipoprotein Class	Osmium μg / Lipoprotein μg	
	1/2 hr	4 days
S_f 20-10^5	0.624	1.03
S_f 0-20	0.245	0.231
HDL	0.380	0.421
Albumin	0.047	0.391

(triglyceride, phospholipid, cholesterol ester, etc.) and fatty acids present in these lipids. They present the material to be reacted with osmium tetroxide in a macro-molecular form and represent a naturally occurring complex with varying lipid-protein proportion.

The lipoproteins were isolated from human serum using an ultracentrifugal technique (5,6) essentially as previously described. For this study three lipoprotein classes and centrifugal isolated albumin were studied: low-density (S_f 20-10^5), intermediate-density (S_f 0-20) and high-density lipoprotein (HDL). Lipoprotein concentrations, determined by analytic ultracentrifugation, ranged from 0.311% to 2.38% lipoprotein. 0.15 ml of lipoprotein solution was placed in 0.5 ml of buffered (phosphate buffer, pH 7.4) 1% osmium tetroxide and kept at room temperature for either half an hour or 4 days. 0.01 ml was then removed, dried sufficiently for the excess osmium tetroxide to evaporate and the osmium content assayed by the X-ray fluorescence technique as developed by Gofman (7). All samples were prepared for osmium analysis by evaporation of aliquots of solutions into a specific region on identical filter-paper disks. The disks were supported in lucite holders of dimensions standard for the Siemens X-ray fluorescence apparatus utilized. Analyses for osmium were made on the $L\beta_1$ line (γ = 1.197Å), with the following operating parameters: 50 kV, 38 mA, LiF monochromator, scintillation detector. Yields were 215 counts/min/μg osmium. Control samples of the ultracentrifugal salt background in buffered osmium tetroxide were run and showed that essentially no osmium was present after evaporation.

RESULTS

Table 1 shows the osmium content of the lipoprotein classes and albumin after reaction with buffered 1% osmium tetroxide for half an hour or 4 days. The results are given in terms of weight of osmium in μg per μg of lipoprotein. It can be seen that lipoproteins of S_f 0-20 and HDL have essentially completed the osmium uptake in half an hour and add little or nothing in the next 4 days. Albumin on the other hand is quite slow in reacting with osmium tetroxide and

Table 2.

Lipoprotein class	Molecular weight	% Protein	% Lipid	Fatty acid double bond per molecule
S_f 20-10^5	1 x 10^7	12%	88%	21,000
S_f 0-20	3 x 10^6	23%	77%	5,200
HDL	220,000	50%	50%	280
Albumin	70,000	99.8%	0.2%	0.5

shows a five-fold increase at 4 days compared to the half-hour value. The very low-density S_f 20-10^5 class shows the greatest osmium uptake, an amount equal to the weight of lipoprotein itself. The S_f 20-10^5 lipoprotein rapidly takes up large amounts of osmium but continues to add osmium during the 4-day phase. This may be due to large size of the particles and consequent lengthening of the time necessary to penetrate these largest of the serum lipoproteins.

Table 2 shows the physico-chemical data on these lipoprotein classes as determined by techniques previously reported (6). Also shown is an estimate of the number of double bonds contained in fatty acid in each molecule. Such an estimation is based on fatty-acid composition determined by gas chromatography (8). The distribution of osmium between the classes indicates that the osmium tetroxide reacts with the unsaturated fatty acid of the molecule rather than with the protein or saturated lipid. The amount of osmium taken into the molecule can be accounted for by about one osmium atom at each of the ethylenic double-bond sites.

DISCUSSION

There are several observations supporting these data. First the uptake of osmium compounds was checked by weighing the osmium sample after evaporating off the volatile excess osmium tetroxide. Since we have shown by X-ray fluorescence studies that no osmium is present in the control osmium-tetroxide-salt sample, only the osmium compounds formed by reaction with the lipoproteins would add to the weight of the sample. This analysis was performed on lipoproteins after one week of exposure to 1% buffered osmium tetroxide and showed an uptake of osmium compounds equal to the weight of lipoproteins and double the weight of albumin. Considering the very long exposure to osmium tetroxide the high uptake by albumin is not surprising.

The mean density of the natural S^o_f 0-20 is approximately 1.035 g/ml. After reaction with osmium tetroxide the density was determined using capillary

pycnometer, sedimentation equilibrium, and hydrostatic weighing techniques. The density of the osmiated molecule was found to be 1.84 g/ml. The change in density can be used to calculate the amount of osmium added to the molecule. This calculation again gives the result that after long-term exposure to osmium tetroxide the amount of osmium compounds added is equal to the weight of the molecule itself. This method is free of the error that would be introduced by the presence of osmium compounds not attached to the molecule itself.

Finally, extraction of the lipid from lipoproteins by a modified Sperry-Brand technique (9) after interaction with osmium tetroxide yields the expected amount of saturated fatty acids as determined by gas chromatography but almost none of the unsaturated fatty acids found in the untreated molecule. This would again indicate that the unsaturated lipids have reacted with the osmium, and have been rendered insoluble.

These results would indicate that the uptake of osmium is determined by the amount of unsaturated fatty acid present in the molecule, and that the protein moiety and saturated fatty acids contribute very little to this reaction at least at half an hour reaction time. Since most fixation times for tissue for electron microscopy are on the order of half an hour, this would support the views of Wigglesworth (3) and Hess (4) that the distribution of osmium in fixed tissue is determined chiefly by the distribution of unsaturated lipid.

SUMMARY

A quantitative measurement of the uptake of osmium by lipoprotein has been made using an X-ray fluorescence technique to analyze for osmium. Results show osmium tetroxide reacts primarily with double bonds of unsaturated lipids.

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Influence of the Thymus on the Output of Thoracic-Duct Lymphocytes

John C. Schooley and Lola S. Kelly

The thymus is a complex organ containing lymphocytic, reticular and epithelial cells. Besides, by the presence of numerous epithelial cells, the thymus is distinguished from other lymphoid tissue by the presence of Hassall's bodies and by the absence of true lymphatic nodules, of well-developed lymph sinuses and of afferent lymphatics. The morphological and functional relationships between the lymphocytic cells of the thymus and of the lymph nodes have been controversial for almost half a century. Recent embryological studies suggest that lymphocytes of the thymus are of endodermal origin, whereas lymphocytes of the lymph nodes are of mesodermal origin (1). This difference in origin has been suggested to impart to these cells differences in behavior and in function (2). Large cells with basophilic cytoplasm, with fine reticular nuclei and giant nucleoli, termed "lymphogonia" by Amano (3), are rarely found in the thymus but are frequently observed in other lymphoid tissues. Cytological (4) and autoradiographic observations (5) indicate that the small lymphocyte of the thymus, in contradistinction to the small lymphocyte of the lymph nodes, is a dividing cell. These differences, and others which will be more thoroughly discussed, suggest that the thymus may play a unique role in the physiology of lymphoid tissue and in the ontogeny of immune responses.

Various effects of removal of the thymus have been studied; the older literature has been reviewed by Crotti (6). The newer concepts of the physiology of lymphoid tissue and of the life history of the lymphocyte, as well as current immunological speculations, have resulted in a renewed interest in the effects of thymectomy. The recent work of many investigators has demonstrated that neonatal thymectomy causes: (a) a decreased hourly output of lymphocytes in thoracic-duct lymph (7,8); (b) a diminished lymphocyte population in blood and in lymph nodes (9,10,11); (c) a severe depression in immunological responsiveness (12,13,14,15,16); and (d) a progressive wasting or runting disease (13,17). The experiments reported here deal primarily with the decreased output of thoracic-duct lymphocytes and with the decreased lymph-node development following thymectomy. Brief reports of some of this work have appeared previously (8,18).

MATERIALS AND METHODS

Groups of specific-pathogen-free (SPF) or of conventional Long-Evans rats

were used. Rats were thymectomized or sham-operated at either 6-8 days or 30 days of age by the use of deep hypothermia and ether anesthesia respectively. The completeness of thymectomy was determined at autopsy. Animals containing residual thymus were discarded.

The hourly output of lymphocytes in the thoracic-duct lymph was determined in anesthetized animals (7 mg sodium pentobarbital/100 g body wt) 60 days after thymectomy or after sham-operation. The thoracic duct was cannulated in the neck by using the procedure described by Reinhardt (19). In one experiment rats were anesthetized, and the thoracic duct was cannulated simultaneously in the neck and the abdominal cavity, using the method of Bollman, Cain and Grindlay (20). Lymph was collected from the cannulated ducts for at least 1.5 hr, and its volume was measured. Total white-cell counts were made of the lymph in duplicate; all cells observed in the counting chambers were considered to be lymphocytes. The results are expressed as the hourly output of lymphocytes per 100 g body weight.

Animals whose thoracic ducts had not been cannulated were anesthetized with ether and exsanguinated via the abdominal aorta before autopsy. The spleen, thymus (of sham-operated animals), and various groups of lymph nodes were dissected out, cleaned of fat and adjacent tissue, and weighed. The following groups of lymph nodes were weighed separately: mesenteric, axillary, brachial, mediastinal, inguinal, and superior and deep cervical. The total weight of all these representative lymph nodes was calculated. The results are expressed as milligram of lymph node per 100 g body weight. Animals with evidence of respiratory or enteric disturbances were discarded at autopsy.

The DNA specific activities of lymph nodes and spleen of another group of conventional rats were determined 60 days after thymectomy or after sham-operation at 6-8 days of age. DNA was isolated, by the method of Schmidt and Thannhauser (21), from the spleen and from a pooled sample of lymph nodes of each rat 3 hr after the intraperitoneal injection of 40 μC of $Na_2HP^{32}O_4$. An aliquot was removed for determination of total DNA phosphorus. The remaining DNA was reprecipitated two times before the determination of specific activity.

The effect of subtotal removal of the mesenteric lymph nodes on the hourly output of lymphocytes from the thoracic duct was determined. The mesenteric nodes found lying in a row along the root of the mesentery were removed after incision of the overlying peritoneum in ether-anesthetized 30-day-old SPF animals. The same general procedure was used for the sham-operations. Thirty days later the thoracic-duct lymph was collected, the thoracic-duct-lymphocyte output calculated, and the lymph nodes weighed.

$C_{57}L$ mice were parabiosed by a skin union without coelio-anastomosis.

Three months after the operation one member of the parabiont was injected intravenously with 15 μC of tritiated thymidine (1.9 C/mM) every other day for a total of three injections. One day after the last injection of tritiated thymidine, smears were made of blood taken from the tip of the tail of each animal and of lymph collected from the thoracic ducts. Autoradiographs were made of the smears and of sections of various lymph nodes with Kodak Ar-10 stripping film. The autoradiographs were processed and scored as previously described (22).

The effects of injection of thymus and lymph-node extracts on the lymph-node weights of SPF rats thymectomized or sham-operated at 6-8 days of age were determined. The extracts were prepared as described by Metcalf (23) from the tissues of SPF male rats weighing about 100 g apiece. Each extract contained the equivalent of 224 mg of tissue per ml. Groups of rats were injected immediately after the operations and then three times weekly for 8 weeks with 0.1 ml of the extracts subcutaneously in the back of the neck. The extracts each animal received weekly were equivalent to 67.2 mg of the respective tissues. Control animals were similarly injected with saline. Aliquots of the extracts were kept frozen until used.

The primary and secondary agglutinin response to antigens of SPF rats thymectomized or sham-operated at either 6-8 or at 30 days of age were compared. The antigen used was Salmonella adelaide (SW. 1338; XXXV: f.g.). A maximally turbid liquid culture of bacteria was killed by the addition of benzalkonium chloride (Roccal) to a final concentration of 1:5000, centrifuged, washed once, and suspended in saline to a final concentration of about 10^9 organisms per ml. All four groups of rats were injected with 0.1 ml of antigen into the tail vein when they were five months old. Blood was withdrawn from the tail of one-half of the members of each group every other day for 15 days. The serum was removed and frozen. A similar series of rats was reinjected with the antigen at 10 months and blood samples were collected. The agglutinin titers of the various sera were determined by the test-tube agglutination technique of using formalin-killed cells suspended in saline and doubling dilutions of the serum; the initial dilution was 1:10. The titer was defined as the $\log_2$ of the reciprocal of the highest dilution of serum giving a macroscopic agglutination.

The various experiments presented here were performed during the last 4 or 5 years. The quality of the experimental animals, particularly the specific pathogen-free animals, has varied during this time. Consequently, comparisons of lymph-node weights of animals in the different experiments are not necessarily valid. The controls for each separate experiment were performed, however, at the same time and under the same experimental and environmental conditions.

RESULTS AND DISCUSSION

The effect of neonatal thymectomy on thoracic-duct-lymphocyte output,

Table 1. Effect of neonatal thymectomy on the output of lymphocytes in the thoracic duct lymph and blood lymphocyte levels

Treatment	No. of Animals	Animal wt	Lymph volume mm^3/hr	Lymphocytes/ mm^3 of lymph	Lymphocyte output millions/hr/100 g body wt	Lymphocytes/ mm^3 of blood
Conventional sham-operated 6-8 days old	12	207 ± 16*	640 ± 62	38,800 ± 4,500	11.7 ± 1.4	17,500 ± 1,500
Conventional thymectomized 6-8 days old	11	196 ± 15	623 ± 52	10,190 ± 1,200	3.10 ± 1.0	10,500 ± 1,000
Specific pathogen-free	14	249 ± 4	449 ± 29	30,350 ± 1,980	5.34 ± 0.54	8,330 ± 650

*Standard error of the mean.

lymph flow, and blood lymphocytes is shown in Table 1. The thoracic-duct-lymphocyte output of the thymectomized animals was about 27% of that found in sham-operated controls. This decrease was due to the low concentration of lymphocytes in the lymph; lymph flow was not significantly altered. Bierring (24) found that after thymectomy of young adult rats, the lymphocyte output was decreased to about 60% of that found in control animals. This finding has been confirmed recently in this laboratory (8). Reinhardt and Yoffey (7) observed a decreased lymphocyte output 40 days after thymectomy of young adult guinea pigs, but a considerable reduction in thoracic-duct-lymph flow occurred.

If the thymus, as a major lymphocytopoietic organ, were to contribute large numbers of lymphocytes directly to the thoracic duct, then a reduction in the output of lymphocytes in thoracic-duct lymph would be expected following thymectomy. Kindred (25) assumed that thymic lymphocytes entered the blood stream via the thoracic and right lymphatic ducts. Several lines of evidence suggest, however, that only insignificant numbers of thymic lymphocytes pass into the blood stream by these routes. Mann and Higgins (26) found that the lymphocyte output from intestinal lymphatics was almost equal to the lymphocyte output from the thoracic duct. Hungerford and Reinhardt (27) found no significant difference in the lymphocyte outputs of similar groups of rats when thoracic-duct lymph was collected either from the jugular lymph sac in the neck or from the duct in the abdominal cavity. The results of simultaneous collection of lymph from these two sites in the same animal, shown in Table 2, indicate that not more than 10% of the thoracic-duct lymphocytes come from lymphoid tissue within the thoracic cavity. The low lymphocyte output of the right lymphatic duct (28) suggests that, in the rat, this route for the discharge of thymic lymphocytes into the blood is insignificant. Furthermore, direct connection of the lymphatic channels of the thymus with the thoracic duct cannot be demonstrated after injection of trypan blue directly into the thymic parenchyma, although trypan blue does enter the blood. Sainte-Marie and Leblond (29) conclude from morphological studies that small lymphocytes leave the medulla of the thymus by entering the blood and lymphatic circulation. If the thymic lymphocytes do not leave the thymus, the marked reduction in output of thoracic-duct lymphocytes after thymectomy is difficult to explain. We assume, for the present, that thymic lymphocytes do migrate from the thymus; most of them do so apparently either by passing directly into the blood stream or by entering unknown lymphatic routes which do not terminate with the thoracic or right lymphatic ducts. In any case, the decreased output of lymphocytes in the thoracic-duct lymph after thymectomy cannot be simply the result of the removal of a major lymphocytopoietic organ that contributes lymphocytes directly to the thoracic duct.

An explanation for much of the decreased output of thoracic-duct lymphocytes after thymectomy can be based on the recent experiments of Gowans (30) and Shorter and Bollman (31). If, as they propose, large numbers of lymphocytes

Table 2. Lymph flow, cell content, and cellular output of abdominal and neck thoracic duct lymph

	Lymph volume mm^3/hr	Lymphocytes/ mm^3 of lymph	Lymphocyte output millions/hr	Lymphocyte output millions/hr/ 100 g body wt
Abdominal thoracic duct	368 ± 18*	32,800 ± 1,030	11.3 ± 1.42	4.22 ± 0.54
Neck thoracic duct	124 ± 5.5	5,060 ± 920	0.64 ± 0.12	0.23 ± 0.04

*Standard error of the mean.

recirculate from the blood and from tissues to the thoracic-duct lymph, then the thoracic-duct-lymphocyte output could be a reflection of the total lymphocyte pool. The differences observed in the thoracic-duct-lymphocyte outputs and in the mass of organized lymphoid tissue of intact conventional and intact specific-pathogen-free rats are consistent with this concept (Tables 1 and 4). Removal of a highly proliferative tissue, such as the thymus, should decrease the total lymphocyte pool, and thus indirectly decrease the thoracic-duct-lymphocyte output. Yoffey (32) has questioned the conclusions of the experiments on lymphocyte recirculation and suggests that the use of immobilized rats may have influenced the results. We have, therefore, attempted to corroborate these experiments on recirculation of lymphocytes by using two different approaches where immobilization of the experimental animals was unnecessary.

The mesenteric lymph nodes are frequently considered a major source of thoracic-duct lymphocytes. The effects of subtotal removal of the mesenteric lymph nodes on the flow, lymphocyte content, and lymphocyte output of thoracic-duct lymph are shown in Table 3A. The weights of the remaining representative lymph nodes are shown in Table 3B. The removal of almost 75% of this mass of lymphoid tissue failed to decrease significantly the thoracic-duct-lymphocyte output, suggesting that the mesenteric lymph nodes are not necessarily the ultimate source of thoracic-duct lymphocytes. These results favor the concept of lymphocyte recirculation, especially since hyperplasia of other lymphoid tissue draining directly into the thoracic duct was not observed.

The rapid disappearance of H^3-thymidine after intravenous injection, compared with the slow rate of blood exchange through the skin capillaries of parabiosed animals, suggested that the DNA-synthesizing cells of only one member of a parabiont would be labeled. After injection of H^3-thymidine into one partner, none of the rapidly dividing, intestinal epithelial cells of the noninjected partner was labeled; whereas, essentially every cell of the injected partner was labeled (Fig. 1). This result indicates that very little H^3-thymidine passed from the injected to the noninjected partner of the parabiont. Thus, the only source of labeled cells, such as lymphocytes, in the noninjected partner would be cells produced in the labeled partner. The percentage of labeled lymphocytes varied in the blood and in the thoracic-duct lymph of the labeled and nonlabeled partners of different parabionts. However, in each individual animal the same percentage of labeled lymphocytes was found in the blood and in the thoracic-duct lymph.

For example, in one parabiont the percentage of labeled lymphocytes in the blood and in the thoracic-duct lymph of the H^3-thymidine-injected partner was about 18%; whereas, about 9% of the lymphocytes were labeled in the blood and in the thoracic-duct lymph of the noninjected partner. The labeled lymphocytes found in the thoracic-duct lymph of the noninjected partner were presuma-

Table 3. Effect of mesenteric lymph node removal on thoracic duct lymphocyte output and lymph node weights

A

	No. of animals	Lymph volume mm^3/hr	Lymphocytes/mm^3 of lymph	Lymphocyte output millions/hr/100 g body wt
Mesenteric lymph nodes removed	10	490 ± 27*	51,380 ± 4,030	8.11 ± 0.68
Sham-operated	10	465 ± 28	42,800 ± 3,650	6.82 ± 0.72

B

	Body wt	Lymph node weights (mg/100 g body wt)								
		Mesenteric	Axillary	Brachial	Mediastinal	Superior cervical	Deep cervical	All nodes	Spleen	Thymus
Mesenteric lymph nodes removed	301 ± 6.7*	18.6 ±2.2	18.3 ±1.7	17.4 ±1.3	23.4 ±5.1	68.7 ±5.2	35.8 ±3.3	212 ±16.0	226 ± 9.5	165 ±13.8
Sham-operated controls	291 ± 7.8	70.2 ±5.4	18.6 ±1.5	17.7 ±1.5	21.8 ±4.9	70.0 ±5.2	36.8 ±7.5	263 ±16.2	190 ± 9.8	169 ±11.2

*Standard error of the mean.

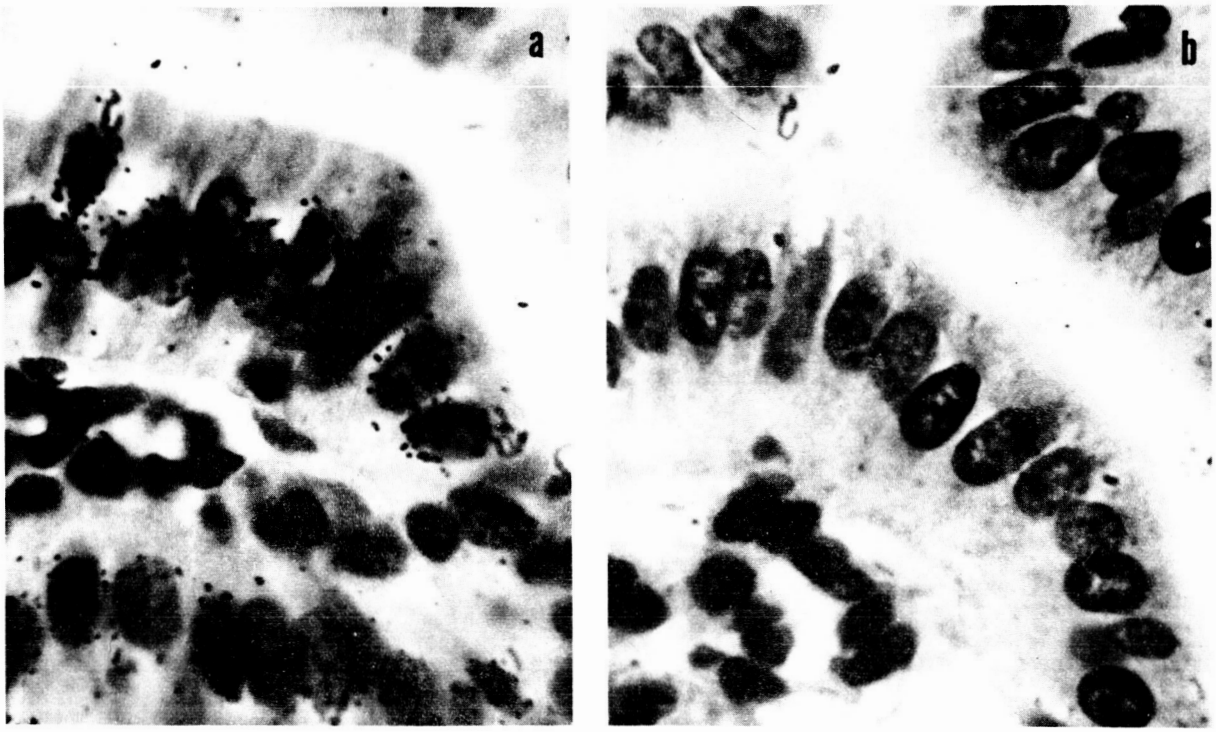

Figure 1. Autoradiographs of the intestinal epithelium of parabionts after the multiple injection of H^3-thymidine to one partner. A. Injected partner. B. Noninjected partner.

bly derived from the blood of the noninjected partner. The similar labeling of lymphocytes in the blood and in the lymph of the noninjected partner favors extensive lymphocyte recirculation.

We suggest that the thymus contributes significant numbers of lymphocytes to the total recirculating-lymphocyte pool. Removal of the thymus removes this source of cells and thus decreases the number of recirculating lymphocytes. This decrease is then reflected in a decreased output of thoracic-duct lymphocytes. Moreover, some data (33) suggest to us that, although the thymus is a source of recirculating lymphocytes, none of these cells recirculates through the thymic parenchyma.

The marked reduction in thoracic-duct-lymphocyte output after neonatal thymectomy cannot be entirely accounted for on the basis of the removal of cells that would be produced by the adult thymus. As we have previously reported (8), the development of cervical and mesenteric lymph nodes is influenced by neonatal thymectomy, as seen in a 20-30% decrease in lymph node weight. Similarly, Reinhardt (9) had noted that these lymph nodes weighed significantly less than those in sham-operated controls 63 days after neonatal thymectomy. Bierring (24) however, did not observe such a difference in lymph-node weight of rats thymec-

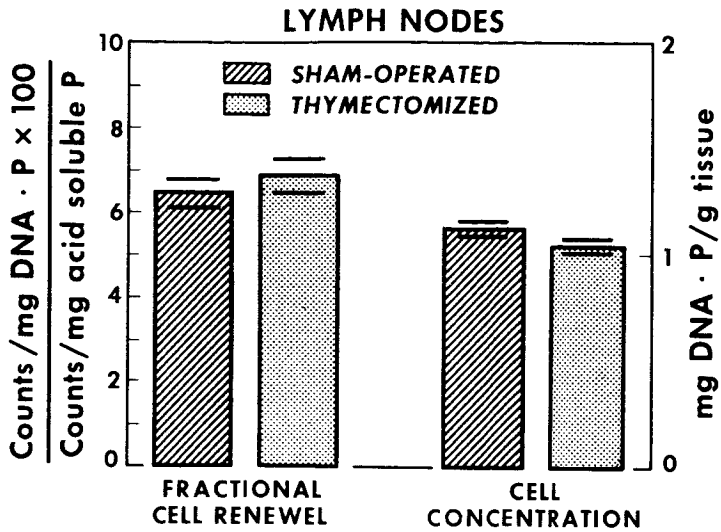

Figure 2. DNA synthesis and DNA concentration in pooled lymph nodes of neonatally thymectomized or sham-operated rats. Fractional cell renewal: DNA specific activity as percent of acid-soluble specific activity 3 hours after injection of $Na_2HP^{32}O_4$. MU-28370

tomized at 50-60 days of age. Manning (34) found only a slight reduction, of doubtful significance, in the lymph-node weights of Wistar rats 12-15 weeks after neonatal thymectomy. She did find, however, highly significant differences in the lymph-node weights when thymectomized and sham-operated animals were compared after injection with ACTH for 10 days.

Because of these variable findings, we have restudied the problem of inadequate development of lymph nodes following thymectomy at different ages. The results of these experiments are given in Table 4. Values for specific-pathogen-free animals are given for comparison. Neonatal thymectomy results in a significant reduction ($P < 0.001$) in the total weight of the representative lymph nodes in 68-day-old animals. The differences seen in the lymph-node weights of individual groups of lymph nodes are significant ($P < 0.01$-0.001) except for the mediastinal and deep cervical nodes. In agreement with Bierring (24) the lymph-node weights of rats thymectomized at 30 days of age were not significantly different from sham-operated controls. The lymph-node weights of the neonatal-thymectomized rats, although significantly less than the controls, were still much higher than the lymph-node weights of SPF rats of the same age. This suggests that active lymphocytopoiesis occurs in the lymph nodes of rats thymectomized at 6-8 days of age despite the decreased lymph-node weights.

The effects of neonatal thymectomy on lymphocytopoiesis, as measured by DNA specific activities after injection of P^{32}, are shown in Fig. 2. Neither the fractional cell renewal nor the cell concentration (DNA phosphorus per gram tissue) in the nodes of thymectomized rats was significantly different from the nodes of sham-operated controls. These results suggest that the thoracic-duct-lymphocyte output of neonatally thymectomized rats is influenced by the decreased amount of lymphoid tissue rather than by a decrease in the proliferative

Table 4. Effect of thymectomy at different ages on lymphoid tissue

Treatment	Body wt	Lymph node weights (mg/100 g body weight)								Spleen	Thymus
		Mesenteric	Axillary	Brachial	Mediastinal	Inguinal	Superior cervical	Deep cervical	All nodes		
Sham-operated 6-8 days old. Autopsied at 68 days (12 animals)	272 ±10.9*	81.8 ±3.4	63.3 ±3.4	45.1 ±3.7	22.4 ±1.6	40.2 ±2.6	128.5 ± 7.5	43.8 ±4.1	425 ±15.2	476 ±64.0	173 ±10.3
Thymectomized 6-8 days old. Autopsied at 68 days (14 animals)	245 ± 7.8	54.9 ±2.4	47.4 ±3.6	28.5 ±2.1	26.4 ±3.5	26.4 ±1.9	85.7 ± 5.1	38.6 ±2.9	308 ±12.4	425 ±36.1	
Sham-operated 30 days old. Autopsied at 90 days (10 animals)	307 ±10.1	75.6 ±3.1	36.9 ±3.1	27.6 ±3.1	19.5 ±3.2	33.7 ±2.7	91.6 ± 6.9	30.5 ±3.0	315 ±17.9	397 ±26.2	91.4 ± 5.4
Thymectomized 30 days old. Autopsied at 90 days (13 animals)	329 ± 8.6	65.3 ±2.2	34.1 ±2.5	24.7 ±2.1	21.0 ±4.1	28.0 ±1.8	78.8 ± 4.9	23.7 ±1.5	276 ±13.8	436 ±30.5	
Specific pathogen-free. Autopsied at 68 days (16 animals)	250 ± 3.9	49.6 ±3.0	16.3 ±1.0	16.7 ±0.9	12.4 ±1.3	17.1 ±0.9	43.6 ± 1.6	13.7 ±1.1	169 ± 5.4	176 ± 5.6	169 ±13.2

*Standard error of the mean.

Table 5. Effect of thymus and lymph node extracts on the lymphoid tissue of neonatal-thymectomized SPF rats

Treatment	Body wt	Lymph node weights (mg/100 g body weight)								Spleen	Thymus
		Mesenteric	Axillary	Brachial	Mediastinal	Inguinal	Superior cervical	Deep cervical	All nodes		
Sham-operated saline injected (9 animals)	272 ± 5.4*	114 ± 5.5	24.3 ±2.0	21.7 ±1.3	22.1 ±1.7	22.7 ±1.9	93.9 ±11.2	41.5 ±4.7	341 ±18.4	196 ± 7.9	202 ±14.2
Thymectomized saline injected (10 animals)	296 ±11.9	64.9 ± 4.9	15.4 ±1.2	12.1 ±1.3	20.9 ±4.0	13.5 ±1.3	75.4 ±5.2	29.9 ±2.9	232 ±12.8	198 ±14.3	
Thymectomized injected with thymus extract (14 animals)	270 ± 4.3	77.4 ±4.1	24.3 ±3.4	17.9 ±2.1	53.2 ±5.3	17.6 ±1.4	97.5 ±5.3	62.8 ±4.6	351 ±13.5	344 ±22.5	
Thymectomized injected with lymph node extract (14 animals)	273 ± 6.7	68.1 ±3.7	19.2 ±1.0	17.1 ±1.2	48.3 ±6.5	14.6 ±0.9	95.6 ±8.4	59.9 ±4.9	331 ±22.6	401 ±39.9	
Auto-transplant (8 animals)	310 ±14.5	63.3 ±5.3	17.2 ±1.3	14.3 ±1.4	19.1 ±3.2	16.7 ±1.8	76.7 ±5.0	25.2 ±2.6	232 ±16.7	170 ±11.6	

*Standard error of the mean.

All animals autopsied at 68 days of age.

capacity of the remaining lymphoid tissue. Bierring (24) observed no change in mitotic activity in lymphoid organs of rats thymectomized at 50-60 days of age. The poor development of lymphoid tissue following neonatal thymectomy compared to the apparently normal development following thymectomy of one-month-old rats implicates the thymus in the development of lymphoid tissue primarily in the neonatal animal.

The presence of a lymphocytosis-stimulating factor in the thymus has been demonstrated by Metcalf (23) in mice. He has suggested that this factor is concerned in the normal control of lymphocytopoiesis (35). The DNA specific activities and the weight of the lymph nodes of thymectomized rats indicate that the thymus of young animals is not indispensable for lymphocytopoiesis. However, the poor development of lymphoid tissue after neonatal thymectomy suggests that the thymus may supply a humoral factor specifically required in the neonatal animal, or the thymus may supply cells which assist in populating the lymph nodes.

The effects of thymus and lymph-node extracts on the development of the lymph nodes of SPF neonatal-thymectomized rats are shown in Table 5. A significant reduction in the lymph-node weights was found although the lymph nodes of these animals normally weigh less than the lymph nodes of conventional animals. This reduction was not observed when the animals received extracts of either thymus or lymph node. An increase in the weight of the superior and deep cervical groups of lymph nodes was responsible for most of the observed increase in the total weight of the representative lymph nodes of neonatal-thymectomized SPF rats. The deep-cervical-lymph-node group weighed significantly more ($P < 0.001$) than that of nonthymectomized controls. These cervical groups of nodes presumably received more contact with the extracts because of the routes of absorption from the subcutaneous injection site. Thymectomy did not alter the spleen weights, whereas, interestingly, a significant increase in spleen weight occurred following injection of both extracts. Histological observations indicate that the increased spleen weight after injections of both extracts was not confined to white pulp. Precipitins against the extracts could not be demonstrated in the serum of the injected animals with agar-diffusion methods. Further work is obviously necessary, and any interpretation of these data must be speculative in view of the fact that the increased lymph-node weights may be the result of many unknown factors. Quantitative measurements of changes in the relative numbers of different types of lymphocytic cells in the lymphoid tissues after thymectomy are required. If the observed effects on lymphoid tissue are the result of a proliferative response, the factor responsible for this response is not confined exclusively to the thymus of the young weanling rats whose tissues were used for preparing the extracts. The cells responsible for producing this factor could have come initially from the thymus.

The significance of living thymic cells in the development of lymphoid

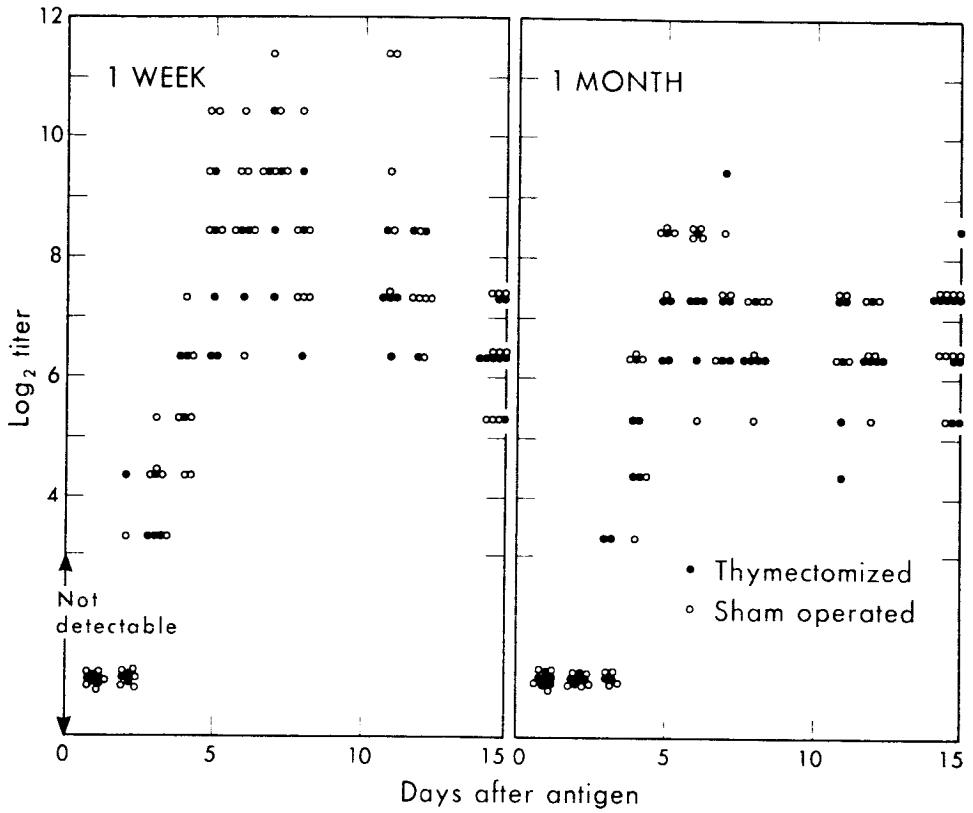

Figure 3. Agglutinin titers to Salmonella adelaide in rats thymectomized or sham-operated at 6-8 days or 30 days of age.
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tissue in neonatally-thymectomized rats was investigated by using autotransplants of thymus. These thymic autotransplants apparently did not grow as thymic tissue was not observed at autopsy. The lymph-node weights of the thymectomized-thymus-autotransplanted group of rats were indistinguishable from those of the thymectomized group of rats (Table 5). The failure of the autotransplants to grow may be simply the result of the trauma involved in this operation or may be related to the stage of lymphoid tissue development at the time of autotransplantation. Miller (13) presents evidence indicating that growth of neonatal thymuses does occur after transplantation into 7-to-14-day-old neonatally-thymectomized mice. Other data on the effects of age on autotransplantation of the thymus in newborn rats and mice are not available.

The production of antibody by lymphoid tissue is well established, although controversy exists concerning the significance of the various types of lymphocytic cells in different types of immune responses. An alteration in immunological responsiveness might be expected after neonatal thymectomy because a marked reduction in total lymphoid tissue exists. The primary agglutinin response to Salmonella adelaide of rats thymectomized at 6-8 or 30 days of age is given in Fig. 3. The temporal pattern of agglutinin titers of the thymectomized

rats was indistinguishable from sham-operated controls, irrespective of the age at thymectomy. Furthermore, the secondary response of these different groups of thymectomized and sham-operated animals was identical.

Jankovic, Waksman, and Arnason (16) have found other immune responses to be impaired in rats thymectomized at 1-5 days of age. Miller (13) found the primary agglutinin response to Salmonella typhi H impaired in mice after thymectomy at birth. The relation between the severity of immunological defects after thymectomy and the age of the animal at thymectomy suggests a thymic role in the ontogeny of at least some immune responses. This role of the thymus is not necessarily related to the role of the thymus in the development of lymphoid tissue. The age dependency of the effects of thymectomy on immunological reactions is not in conflict with the hypothesis that the thymus is the ultimate source of immunologically competent cells (36). These results do suggest, however, that after several weeks of life the release into the circulation of immunologically competent stem cells from the thymus is not necessary for the maintenance of normal immunological responses. After severe involution of lymphoid tissue the thymic source of immunologically competent cells may be required to restore normal immunological responsiveness.

SUMMARY

Thymectomy of rats at 6-8 or 30 days of age results in a decreased output of lymphocytes from the thoracic duct. This decreased output is not simply the result of the removal of a lymphoid tissue which discharges lymphocytes directly into the thoracic duct. We suggest that lymphocytes produced in the thymus enter a recirculating-lymphocyte pool, and that removal of the thymus decreases this pool. The decrease in the recirculating-lymphocyte pool is then reflected in a decreased output of lymphocytes from the thoracic duct.

Thymectomy of 6-8-day-old rats results in a decrease in the weights of the lymph nodes; whereas, no significant difference in these weights is found in rats thymectomized at 30 days of age. The proliferative activity of this decreased amount of lymphoid tissue is similar to that found in an equivalent amount of lymphoid tissue from sham-operated animals. Thus, a greater reduction in the total lymphocyte pool and in the output of lymphocytes from the thoracic duct occurs following neonatal thymectomy than occurs following thymectomy at 30 days of age.

The observation that injection of saline extracts of thymus or lymph nodes increases the lymph-node weights of neonatally thymectomized rats implicates a humoral agent in lymph-node development, but certainly does not exclude a cellular contribution by the neonatal thymus.

A defective production of bacterial agglutinins could not be demonstrated

in rats thymectomized at 6-8 days of age, in spite of the reduction in the mass of lymphoid tissue.

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Thyroid Function in Chicken and Rat. I. The Effect of Iodine Content of the Diet and of Hypophysectomy on Iodine Metabolism in White Leghorn Cockerels

Lawson L. Rosenberg, Mildred K. Dimick and Gilles La Roche

Several groups of investigators have reported recently on thyroid function in birds as revealed by the metabolism of injected radioiodide (1-4). R. C. Ma (5) has described a severe depression of thyroidal-I^{131} uptake caused by hypophysectomy in chickens. The present studies arose from the observation that chickens in our laboratories had high radioiodide uptakes, and that when the chickens were hypophysectomized their thyroids retained this property to a considerable degree one and a half months post-operatively. This finding was reminiscent of the results described by Morton et al. (6), and by Randall and Albert (7) in which the thyroid glands of hypophysectomized rats maintained on low iodine intakes had high I^{131} uptakes. The present report describes some aspects of the thyroid activity of normal and hypophysectomized chickens as measured by various indices of function with both I^{131} and I^{127}, and the effect of iodide supplementation of the stock diet on these indices. Some experiments were performed in rats and are reported as a basis of comparison of thyroid function of birds and mammals.

MATERIALS AND METHODS

ANIMALS AND DIET. White Leghorn cockerels were hypophysectomized at 6 weeks of age and sacrificed 4 to 6 weeks postoperatively. Normal chickens of the same age were used as controls. Hypophysectomy was performed by the parapharyngeal approach as described by Nalbandov (8). Only those birds which showed complete regression of comb were used in the experiments. At autopsy the pituitary site in each bird was examined with the dissecting microscope for possible pituitary fragments. However, no pituitary remnants were found at autopsy in animals selected for use in experiments on the basis of comb regression. In preliminary experiments, four cockerels judged to be completely hypophysectomized on the basis of regression of comb showed no residual hypophyseal tissue when the pituitary site was examined in serial section. The birds were maintained on a stock diet that had the following composition: corn 30%, wheat 13.5%, barley 10%, alfalfa 4%, bran 15%, fish meal 10%, soy bean meal 10%, whey 2.5%, liver meal 2%, limestone 1.5%, bonemeal 1.2%, salt 0.5%, $MnSO_4$ 0.05%, fish oil 0.25%. The iodine content (The Albert L. Chaney Chemical Laboratory, Inc. Also confirmed by our determinations.) of the hen stock diet was 0.03 μg per g and that of the regular stock diet 0.14 μg per g. When an iodine

supplement was used, it was added as potassium iodide at the level of either 1 or 2 μg per g of diet. In one experiment, normal chickens were maintained on the supplemented diet for 6 weeks prior to autopsy. In another series of experiments both normal and hypophysectomized chickens were maintained on the supplement from hatching time, and in still another experiment birds from hens that had been on the supplement for 4 weeks were then maintained on the supplemented diet from hatching until they were sacrificed.

Rats used were of the Long-Evans strain of various ages to be specified. When hypophysectomized rats were used, the pituitaries were removed when the animals were 28 days old. Some were maintained from birth on the chicken stock diet (finely ground and with the addition of 10% fat) or on the stock supplemented with 1 μg of iodide per g of diet. Some were maintained on a modified McCollum Diet I consisting of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1% fish oil, 0.75% NaCl, 1.5% $CaCO_3$ and KI added to give a total of 1 μg iodine per g of diet.

THYROIDAL RADIOIODIDE UPTAKES AND T/S RATIOS. I^{131} was carrier-free NaI from Oak Ridge and was injected either intravenously or intraperitoneally in saline. When carrier iodide was used, this was added to the injection solution as KI. When only I^{131} uptakes or T/S ratios were measured, 1 μC was injected. T/S ratios (I^{131}) were determined 2 hr after a single blocking dose of 60 mg of Tapazole (methimazole) and 1 hr after the injection of 1 μC of I^{131}, both given intravenously. Completeness of the blockade of organification of the iodine was determined by measuring the radioactivity which was precipitated by homogenization of the thyroid gland in 10% trichloracetic acid.

CHROMATOGRAPHIC ANALYSIS OF THYROID TISSUE. The distribution of iodine, both I^{127} and I^{131}, was determined as follows. The chickens, anesthetized with Nembutal at the desired time after injection of 10-20 μC of I^{131}, were decapitated, and after a very brief period of bleeding, the thyroids were removed, trimmed free of fat, weighed, and then homogenized in NaCl-Tris buffer (0.5 ml for the hypophysectomized animal and 1.0 ml for the normal) in small, glass, motor-driven homogenizers. The buffer had the following composition: 0.11 M NaCl, 0.04 M Tris, 0.001 M $MnSO_4$, and 0.001 M Tapazole. Tris was the proton acceptor form, and the solution had a pH of 10 before homogenization of the thyroid. A 20-μl sample of the homogenate was taken for counting to determine the percentage uptake of the gland. The remainder of the homogenate was transferred to a small test tube that contained dry pancreatin (Viobin Viokase Powder 4 N.F. Pancreatin) to make a concentration of 34 mg per ml; addition of the pancreatin brought the pH to 8.4. Two drops of toluene were added, the tube was tightly stoppered, and hydrolysis was allowed to proceed at 37°C for 24 hr. Then a sample of the digest (usually 20 μl) was applied across the width of a three-inch wide strip of Whatman No. 3MM paper. The iodine-containing compounds

separated by ascending chromatography for 15 hr with a collidine--3 N ammonia solvent system (3:1 vol./vol.). The location of the radioactive components on the chromatograms was determined by autoradiography, and the bands were cut from the papers and counted in a well-type scintillation counter to determine the distribution of thyroidal I^{131} and the completeness of its recovery after fractionation by chromatography. Then the distribution of I^{127} was determined by chemical analysis of the same bands. The I^{127} content of a given volume of the thyroid digest was also determined and used to calculate the total thyroidal-iodine content and, in addition, to ascertain the completeness of recovery of the iodine following chromatographic fractionation. Iodine on the papers was determined by a modification (9) of the familiar method which involves the catalysis of the oxidation-reduction of cerric ion and arsenious ion. The chemical iodine analyses were carefully standardized prior to the chromatographic studies by establishing conditions under which known quantities of iodine were completely recovered in the presence of paper. This was done with filter paper, both before and after treatment with the chromatography solvent. Three chromatograms of each thyroid digest were made, two for duplicate determination of I^{127} and I^{131} and one with added authentic markers of MIT (monoiodotyrosine), DIT (diiodotyrosine), thyroxine or triiodothyronine to identify the radioactive components. The markers were detected by spraying with a solution of diazotized sulfanilic acid (10).

PROTEIN-BOUND IODINE IN THE SERUM. Protein-bound iodine and total iodine were determined in the sera of rats and chickens by precipitation with TCA followed by micro-iodine determinations as described above. In addition, the protein-bound iodine of some samples was also isolated by passage of sera through an anion-exchange resin (Ambulite CO45--Type II-OH-form) to remove iodide. Results obtained by this method were identical with those obtained by precipitation with trichloracetic acid. If thyroid glands were studied in the same animals, the blood was taken prior to removal of the thyroid.

RESULTS

1. Endocrine-Organ Weights of the Normal and Hypophysectomized Cockerel. Table 1 shows that 6 weeks after hypophysectomy the thyroid gland of the 3-month-old cockerel on stock diet weighed only one-third as much as that of the normal chicken of the same age, the comb weight was one-thirtieth that of the normal control, and the testes weight was one-fiftieth that of the normal control. The weight of the adrenal glands of the hypophysectomized cockerel was not strikingly less than that of the normal bird. The body weight continued to increase after hypophysectomy. Similar increase in weight of chickens after hypophysectomy was shown by Nalbandov (8) to be attributable to the laying down of body fat.

Supplementation of the stock diet with iodine did not change the organ weights of the cockerels significantly. However, it was found not infrequently

Table 1. The effect of iodine content of the diet and of hypophysectomy on endocrine-organ weights of white Leghorn cockerels (3 months of age)

Diet	Iodide supplement μg per g diet	Kind of animal	Number of animals	Body weight g	Thyroid weight mg	Comb weight g	Testes weight g	Adrenals weight mg
Stock	None	Normal chickens	28	1189 ± 29†	114 ± 6	27.1 ± 1.6	7.1 ± .4	138 ± 6
"	None	$\bar{H}$ chickens	27	941 ± 28	35 ± 2	0.87 ± .07	0.13 ± .01	90 ± 3
"	2 μg from hatch*	Normal chickens	11	1183 ± 33	92 ± 7	25.3 ± 2.4	6.1 ± .5	123 ± 5
"	2 μg from hatch*	$\bar{H}$ chickens	11	918 ± 48	33 ± 3	0.86 ± .09	0.13 ± .01	82 ± 7

* Or from hens on supplemented diet and then maintained from hatch with a supplement of 2 μg I^- per g of diet.

† Standard error of the mean (±).

that individual chickens on the stock diet had highly enlarged thyroids. This was not observed in chickens that received the iodine supplement.

2A. The Effect of Iodine Content of the Diet and of Hypophysectomy on Radioiodide Uptake and on the Histological Appearance of the Thyroid Gland of the Cockerel. Table 2 demonstrates that the normal chicken on the stock diet concentrated 52% of the injected carrier-free I^{131} in its thyroid in 24 hr; previous experiments established that this maximal uptake had already been reached within 5 hr after injection. The percentage uptake of the radioiodide was not decreased when the isotope was given with 50 μg of carrier iodide, the highest level of carrier tested. Hypophysectomized chickens 6 weeks post-operatively had 24-hr uptakes which averaged 13% of the injected radioiodide. This value also had been reached within 5 hr after injection of the I^{131}. In a previous experiment, chickens that had concentrated these same high percentages of injected I^{131} were judged to be completely hypophysectomized after examination of serial sections of the pituitary site.

The thyroids of the intact chickens were hyperplastic; the very high epithelial cells had pale-staining cytoplasm and vesicular, pale-staining nuclei with mitotic figures and the colloid was pale staining and often completely resorbed. The thyroids of the hypophysectomized birds, on the other hand, appeared inactive with squamous epithelium which had small condensed nuclei; the colloid was compact and darker staining.

To test the effect of iodine content of the diet on radioiodide uptake, cockerels were maintained for 6 weeks prior to autopsy on the stock diet supplemented with 1 μg of iodide per g (added as KI), or from hatching time with a supplement of 2 μg of iodide per g. In still other experiments the cockerels were from hens which had been maintained with the iodide supplement for 4 weeks prior to laying; the cockerels after hatching were maintained on the supplement until they were sacrificed. Table 2 shows the striking reduction in the thyroidal radioiodide uptakes that resulted from supplementation of the diet with iodine. This was observed in both normal and hypophysectomized chickens. The supplementation caused a fourfold reduction of thyroidal-I^{131} uptake in the intact cockerel and brought it to 12%. Such a value is within the range of uptakes characteristic of normal rats. Accompanying this change in iodide uptake was the hyperplastic appearance of the gland which changed to a less active one. Supplementation caused a fivefold reduction of the uptake of the gland of the hypophysectomized chicken to the level of 2.5% of the injected radioactivity which is higher than the usual uptake of the hypophysectomized rat maintained with adequate iodine intake (10).

2B. The Effect of Iodine Content of the Diet on Thyroidal-Radioiodide Uptake in the Rat. When male rats were maintained from birth on the chicken

Table 2. The effect of iodine content of the diet and of hypophysectomy on thyroidal radioiodide uptake of white Leghorn cockerels (3 months of age) and the effect of iodide content of the diet on thyroidal radioiodide uptake of male Long-Evans rats (6 months of age)

Diet	Iodide supplement μg per g diet	Kind of animal	Number of animals	Body weight g	Thyroid weight mg	I^{131} uptake	
						Total thyroid % injected dose	mg Thyroid
Stock	None	Normal chickens	33	1418 ± 37†	141 ± 11	52 ± 1	0.37 ± .03
"	None	H̄ chickens	18	931 ± 36	34 ± 3	13 ± 1	0.38 ± .04
"	1 μg for six weeks	Normal chickens	25	1470 ± 34	128 ± 4	19 ± 1	0.15 ± .01
"	2 μg from hatch *	Normal chickens	11	1176 ± 34	92 ± 7	12 ± 1	0.13 ± .01
"	2 μg from hatch *	H̄ chickens	11	918 ± 48	33 ± 3	2.6 ± .3	0.08 ± .01
Stock	None	Normal rats	19	388 ± 9	39 ± 2	52 ± 2	1.39 ± .08
"	1 μg for six weeks	Normal rats	20	373 ± 12	22 ± 1	16 ± 1	0.78 ± .03

*Or from hens on supplemented diet and then maintained from hatch with a supplement of 2 μg I^- per g of diet.

†Standard error of the mean (±).

stock diet and sacrificed at 6 months of age, their average thyroidal uptake of injected carrier-free radioiodide was 52%, which is identical with that of the chickens on stock diet. This is shown in Table 2. When the diet was supplemented with iodide for 6 weeks prior to autopsy the uptakes were reduced to one-third of this value. The thyroid glands of the rats on stock diet weighed twice as much as those of the animals on the supplemented diet.

Another group of rats was maintained on a diet containing approximately 0.1 μg of iodide per g (Diet I without the iodide supplement) and was used as breeding stock for succeeding generations which were maintained on the same diet. Rats of the first generation when hypophysectomized at 28 days of age and then sacrificed one week post-operatively had I^{131} uptakes of 3%. Rats of the second generation had uptakes which averaged 23% when intact and 7% when hypophysectomized and sacrificed 1 week later. Histologically the thyroids of these hypophysectomized rats appeared to be more active than those of hypophysectomized rats on adequate iodide intake or on low-iodide diet for short periods of time.

3. The Effect of Iodine Content of the Diet and of Hypophysectomy on the T/S Ratio in the Cockerel as Determined with I^{131}. Table 3 shows that 2 hr after a single blocking dose of Tapazole and 1 hr after the injection of radioiodide, the thyroid of the normal cockerel on stock diet maintained an I^{131} concentration gradient against the plasma of more than 650 to 1. Such a value is much higher than in a rat maintained chronically on a goitrogenic drug (11,12). No attempt was made to ascertain whether the T/S ratios measured 1 hr after injection of radioiodide were equilibrium values. In the hypophysectomized bird the T/S ratio was half the value in the intact bird. Iodide supplementation of the diet lowered the value in the intact chicken to one-fifth the value on stock diet, and when these supplemented chickens were hypophysectomized, the T/S ratio was between one-half and one-third of the value in the corresponding intact bird. The resulting T/S ratio in the hypophysectomized chickens was of the same order of magnitude as in the hypophysectomized mouse with blocked thyroids (13) and much higher than the corresponding value usually found in the hypophysectomized rat (10,12,14,15).

4A. The Effect of Iodine Content of the Diet and of Hypophysectomy on Circulating-Iodine (I^{127}) Levels in the Serum of the Cockerel. Table 4 shows that in the hypophysectomized chicken on the stock diet the PBI level was only one-fifth the value in the intact bird; total iodine levels were also lower. When the diet of the intact bird was supplemented with iodine the protein-bound-iodine value increased significantly; in the hypophysectomized animal on the supplemented diet the value was one-third of that in the corresponding intact animal. The total circulating iodine in both the intact and hypophysectomized chickens was markedly higher on the supplemented diet than on the stock.

Table 3. The effect of iodine content of the diet and of hypophysectomy on the T/S ratio of white Leghorn cockerels (3 months of age) determined with I^{131}

Diet	Iodide supplement μg per g diet	Kind of animal	Number of animals	Body weight g	Thyroid weight mg	T/S
Stock	None	Normal chickens	7	1370 ± 13†	92 ± 6	668 ± 70
"	None	H̄ chickens	8	1100 ± 40	27 ± 3	333 ± 58
"	2 μg from hatch*	Normal chickens	8	1248 ± 17	99 ± 5	140 ± 14
"	2 μg from hatch*	H̄ chickens	8	1101 ± 39	30 ± 3	56 ± 3

* Or from hens on supplemented diet and then maintained from hatch with a supplement of 2 μg I^- per g of diet.

† Standard error of the mean (±).

Table 4. The effect of iodine content of the diet and of hypophysectomy on serum iodine (I^{127}) levels in white Leghorn cockerels (3 months of age) and the effect of iodine content of the diet on serum iodine levels in male Long-Evans rats (6 months of age)

Diet	Iodide supplement μg per g diet	Kind of animal	Number of animals	Body weight g	Thyroid weight mg	Serum iodine	
						Protein bound μg per 100 ml	Total
Stock	None	Normal chickens	51	1320 ± 34†	134 ± 9	0.51 ± .02	0.71 ± .03
"	None	$\bar{H}$ chickens	11	862 ± 39	29 ± 2	0.11 ± .02	0.47 ± .03
"	1 μg for six weeks	Normal chickens	25	1470 ± 34	128 ± 4	0.59 ± .03	1.95 ± .12
"	2 μg from hatch*	Normal chickens	10	1170 ± 37	94 ± 7	0.87 ± .02	3.47 ± .42
"	2 μg from hatch*	$\bar{H}$ chickens	11	918 ± 48	33 ± 3	0.31 ± .04	3.53 ± .60
Stock	None	Normal rats	18	388 ± 9	39 ± 2	2.1 ± .1	2.4 ± .1
"	1 μg for six weeks	Normal rats	17	380 ± 12	23 ± 1	3.0 ± .1	5.1 ± .1

*Or from hens on supplemented diet and then maintained from hatch with a supplement of 2 μg $\bar{I}$ per g of diet.

†Standard error of the mean (±).

Table 5. The effect of iodine content of the diet and of hypophysectomy on the iodine (I^{127}) content of thyroid glands of white Leghorn cockerels (3 months of age) and the effect of hypophysectomy on the iodine content of thyroids of male Long-Evans rats (6 months of age)

Diet	Iodide supplement μg per g diet	Kind of animal	Number of animals	Thyroid weight mg	Thyroidal I^{127} Total μg	Thyroidal I^{127} mg Thyroid
Stock	None	Normal chickens	30	97 ± 4†	89 ± 4	0.92 ± .04
"	None	$\bar{H}$ chickens	10	39 ± 3	77 ± 8	1.97 ± .10
"	2 μg from hatch*	Normal chickens	8	86 ± 6	310 ± 26	3.61 ± .10
"	2 μg from hatch*	$\bar{H}$ chickens	6	31 ± 3	92 ± 16	2.96 ± .44
Diet I	1 μg	Normal rats	4	10.2 ± .9	11.5 ± 1.4	1.12 ± .13
"	1 μg	$\bar{H}$ rats	4	4.5 ± .3	5.1 ± .4	1.14 ± .10

*Or from hens on supplemented diet and then maintained from hatch with a supplement of 2 μg I^- per g of diet.

†Standard error of the mean (±).

4B. The Effect of Iodine Content of the Diet on Circulating-Iodine (I^{127}) Levels in the Serum of the Normal Rat. Table 4 also shows that intact rats maintained from birth for a period of 6 months on the chicken stock diet had an average circulating-PBI level of only two-thirds that of similar rats on the same diet but with an iodide supplement for the last 6 weeks before autopsy. The total iodine level in the serum of the rats without supplement was only one-half that in the serum of the supplemented rats.

5A. The Effect of Iodine Content of the Diet and of Hypophysectomy on the Iodine (I^{127}) Content of the Thyroid of the Cockerel. Table 5 shows that 6 weeks after hypophysectomy the iodine content of the thyroid gland of the cockerel on stock diet was almost as high as that of normal controls of the same age; the thyroid weight was approximately one-half, and the concentration of iodine was twice that of the glands of the intact animals. Supplementation of the diet with iodine caused a threefold increase of iodine in the thyroid of the intact bird; the gland of the hypophysectomized chicken on supplemented diet, when examined 6 weeks after hypophysectomy, contained only one-third the amount of iodine found in the gland of the corresponding intact animal but the same amount as that of either the normal or hypophysectomized chicken on stock diet. The gland weight after hypophysectomy was only one-third that of normal, and the concentration of iodine per unit weight of thyroid tissue remained constant.

5B. The Effect of Hypophysectomy on the Iodine (I^{127}) Content of the Thyroid of the Rat. The average iodine content of the thyroid glands of normal and hypophysectomized female rats 2 months of age are given in Table 5. The hypophysectomized rats were sacrificed 1 month post-operatively. The thyroids of the hypophysectomized rats weighed one-half as much as those of intact rats and contained approximately one-half as much iodine. Thus, hypophysectomy did not alter the concentration of iodine per unit weight of thyroidal tissue.

6A. The Effect of Iodine Supplementation of the Diet and of Hypophysectomy on the Distribution of I^{127} in the Iodinated Compounds of the Thyroid Gland of the Cockerel and on the Distribution of I^{131} 24 hr after the Injection of Carrier-Free Radioiodide. Table 6 shows that supplementation of the diet with iodide produced a striking change in the relative distribution of iodine (I^{127}) in the iodoprotein of the intact chickens; there was a very marked decrease in the relative percentages of MIT and thyroxine, and a large increase in the percentage of DIT. On the stock diet, hypophysectomy caused a change in distribution (6 weeks post-operatively) very similar to that produced by iodine supplementation of the intact chicken, namely, a large decrease in the relative amounts of MIT and thyroxine and a marked increase in the percentage of DIT. On the other hand, hypophysectomy of the cockerels maintained on the supplemented diet had very little effect on the relative abundances of the iodinated amino acids. The distribution of iodine in the thyroglobulin indicated molar ratios of MIT,

Table 6. The effect of iodine content of the diet and of hypophysectomy on the distribution of thyroidal I^{127} in the iodinated compounds of thyroid glands of white Leghorn cockerels (3 months of age) and on the distribution of I^{131} twenty-four hours after the injection of radioiodide

Diet	Iodide supplement µg per g diet	Kind of animal	Number of animals	24-Hr I^{131} uptake %		Thyroidal iodine distribution						Total thyroidal I^{127} µg
						Origin %	MIT %	DIT %	Thyroxine %	TITN† %	I^- %	
Stock	None	N chickens	17	41 ± 2‡	I^{131}	8 ± 0	38 ± 1	37 ± 1	15 ± 1	---	2 ± 0	
			5		I^{127}	9 ± 1	28 ± 1	39 ± 0	22 ± 1	---	2 ± 0	85 ± 11
"	None	H̄ chickens	14	14 ± 1	I^{131}	7 ± 1	26 ± 3	63 ± 3	1.4 ± 1	---	2 ± 0	
			6		I^{127}	12 ± 1	13 ± 1	65 ± 1	7 ± 0	---	3 ± 0	72 ± 8
"	2 µg from hatch*	N chickens	7	11 ± 1	I^{131}	6 ± 0	27 ± 1	58 ± 1	7 ± 0	---	2 ± 0	
			3		I^{127}	8 ± 0	18 ± 1	61 ± 1	11 ± 0	---	2 ± 0	275§
"	2 µg from hatch*	H̄ chickens	6	2.8 ± .6	I^{131}	7 ± 0	26 ± 3	63 ± 2	0.8 ± .1	---	3 ± 0	
			3		I^{127}	10 ± 0	18 ± 3	63 ± 4	7 ± 1	---	2 ± 0	107§
Diet I	1 µg	N rats	4	9 ± 2	I^{131}	8 ± 0	22 ± 1	54 ± 1	9 ± 1	3 ± 0	4 ± 0	
			4		I^{127}	14 ± 1	18 ± 0	52 ± 1	10 ± 0	3 ± 0	3 ± 1	11.8 ± 2
"	1 µg	H̄ rats	4	0.2 ± .02	I^{131}	7 ± 0	35 ± 0	50 ± 0	1 ± 0	---	7 ± 1	
			4		I^{127}	8 ± 0	25 ± 1	56 ± 1	8 ± 0	---	3 ± 0	5.1 ± 0.4

*Or from hens on supplemented diet and then maintained from hatch with a supplement of 2 µg I^- per g of diet.

†Triiodothyronine.

‡Standard error of the mean (±).

§Standard errors not calculated (only 3 chickens per group).

DIT and thyroxine in intact chickens on the stock diet of 10:7:2 and on the supplemented diet of 12:22:2. In hypophysectomized chickens on stock diet, the molar ratios of these iodinated amino acids were 15:36:2 and on the supplemented diet 20:36:2. In the case of the stock diet, the glands of the normal and hypophysectomized birds contained approximately the same total amount of iodine, the difference in relative distribution of I^{127} reflecting actual differences in the pool sizes of the thyroidal components in the two kinds of birds. With the supplemented diet, the thyroids of the hypophysectomized animals contained only one-third as much total iodine as those of the corresponding normal controls, and the pool sizes of the iodinated amino acids were correspondingly smaller in the hypophysectomized than in the normal animals, even though the relative distribution of iodine remained very nearly the same. No triiodothyronine was detected in any of the chicken thyroids examined. Table 6, in addition to the distribution of I^{127}, shows the partition of radioiodine which occurred in the iodinated components of the thyroid glands 24 hr after the injection of carrier-free I^{131}. The following details are apparent for intact birds on both kinds of diet, indicating that in this period of time the radioiodide had not equilibrated with the thyroidal I^{127}: (a) the percentage of I^{131} in MIT at 24 hr was higher than that of I^{127}; (b) the percentage of I^{131} in DIT at 24 hr was the same as that of I^{127}; (c) the percentage of I^{131} in thyroxine at 24 hr was lower than that of I^{127}. (For a description and discussion of the kinetics of equilibration of injected I^{131} and thyroidal I^{127} see Paper II of this series.) In the hypophysectomized birds the same was true, essentially, for the relative proportions of I^{131} and I^{127} in MIT and DIT but not in thyroxine. Hypophysectomy of cockerels on both kinds of diets caused a tenfold reduction in the percentage of thyroidal radioiodine which appeared in thyroxine in 24 hr. This change is very similar to that reported in hypophysectomized mice by Wollman and Scow (16), in rats after hypophysectomy by Taurog et al. (10) and in hypophysectomized rats in our laboratories. With I^{131} in all cases, recoveries on the paper chromatograms were within two to three percent of 100%; I^{127} recoveries were usually between 90% and 100%. (Paper II shows data pertaining to recovery of I^{127}.) Thyroid homogenates which were chromatographed prior to addition of pancreatin showed only very small amounts of labelled inorganic iodide (0.5% of the thyroidal I^{131}) and no detectable free, labelled, iodinated amino acids. A time course of the pancreatin digestion showed rapid hydrolysis of the iodoprotein to the extent that 76% of the thyroidal radioiodine had appeared in the hydrolysate at 4 hr, 82% at 8 hr, 86% at 12 hr, 90% at 24 hr and 91% at 36 hr. After 4 hr the relative abundances of the I^{131}-labelled amino acids in the hydrolysate did not change when the hydrolysis was continued to 36 hr, even though the percentage of radioiodine in the hydrolysate had increased during this time interval from 76% to 91%. No attempt was made to hydrolyze the material remaining at the origin which still contained 9% of the thyroidal I^{131}; however, in earlier experiments when the pancreatin preparation was new, hydrolysis proceeded to the extent of 97% with only 3% of the total thyroidal I^{131} remaining at

the origin after chromatography, and the relative abundances of the amino acids in the hydrolysates were the same as in the present experiments.

In order to ascertain that the distribution of iodinated amino acids obtained after digestion really represented the composition of the intact iodoprotein and was not an artifact of the digestion procedure, an experiment was done to test the effect of the digestion on authentic I^{131}-labelled amino acids. When authentic labelled MIT, DIT, thyroxine, or I^{131} was added to a digestion mixture of nonradioactive thyroid tissue (either chicken or rat), which contained pancreatin and which was allowed to incubate for 24 hr, the presence of the digestion mixture caused no change in the labelling of the marker nor did it catalyze the labelling of amino acids liberated from the nonradioactive thyroid protein during the hydrolysis.

6B. The Effect of Hypophysectomy on the Distribution of I^{127} in the Iodinated Compounds of the Thyroid Glands of Rats and on the Distribution of I^{131} at 24 hr after Injection of Carrier-Free Radioiodide. Table 6 shows how the relative pool sizes of the thyroidal-iodine (I^{127}) components in the 60-day-old female rat have changed one month after hypophysectomy. The rats were maintained on Diet I. The percentage of thyroidal iodine in MIT increased significantly. The percentage in DIT remained approximately the same, and that in thyroxine decreased. Twenty-four hours after the injection of radioiodide, the I^{131} had equilibrated with the iodine-containing components in the normal animals; in the hypophysectomized rats the percentage of thyroidal radioiodine in the thyroxine pool was approximately one-tenth that of I^{127}, and a concomitant increase in the label in MIT was present. A puzzling finding in the chromatograms of the digests from normal animals was that the material remaining at the origin was twice as rich in I^{127} as in I^{131}. This has never been found to be true with digests of the chicken thyroids.

DISCUSSION

Although the results of most experiments with hypophysectomized rats indicate that very little thyroidal activity persists after removal of the pituitary (10,12,14,15), there is some evidence that under certain conditions the thyroid gland can retain a substantial amount of function in the absence of the pituitary. Wolf and Greep (17) reported that hypophysectomized rats responded to lowered environmental temperatures with thyroid hyperplasia, and Chapman (18) found that hypophysectomized rats with low-iodine intake responded with increased thyroid weight, cell height and vascularity. Halmi (15), Vanderlaan and Caplan (19) demonstrated that low-iodine intake augmented the response of hypophysectomized rats to thyrotrophin. Morton et al. (6) found that rats, which when intact concentrated 60% of injected carrier-free radioiodide in their thyroid glands, had 24-hr uptakes of 10% when hypophysectomized, and 96-hr uptakes of 22%. This colony of rats had been for many generations on a diet that was later

shown to be inadequate in iodine. It seems likely that the very high uptake in these intact rats was an index of thyroids highly stimulated by dietary iodine lack, and that after hypophysectomy an unusually large residuum of activity persisted in the thyroid tissue. However, the time course of uptake was much more gradual in the hypophysectomized rats than in the normal controls making it possible that the high uptakes in the former were a reflection, in part, of a sluggish excretory mechanism or of a conservation of iodide by the kidney (20). Randall and Albert (7) showed that intact rats on an iodine-deficient Remington diet concentrated 36% of injected radioiodide in their thyroids, and hypophysectomized rats on the same diet concentrated 6.5% of the injected I^{131}.

Our observations (discussed in Results 2B) with hypophysectomized rats which had been bred from stock maintained chronically on a low-iodine diet are again evidence that the thyroid gland can, if it has been previously stimulated, retain considerable activity in the absence of the pituitary. The average uptake of hypophysectomized rats of a second generation on the iodine-deficient diet was 7%; this uptake is characteristic of intact animals on an adequate iodine intake. The thyroids of these hypophysectomized rats showed the characteristic defect in the percentage of the thyroidal radioiodine which entered the thyroidal-thyroxine pool (10,16). It is difficult at the moment to assess the physiological importance of the high avidity of these thyroids for iodide. This problem is presently under investigation.

In the case of our intact chickens on stock diet, the hyperplastic appearance of the thyroids, the high thyroidal-radioiodide uptakes, even with 50-μg carrier iodide, and the high T/S ratios are not characteristic of the behavior of thyroids of chickens, *per se*, but were a reflection of the low iodine content of the diet. This was made evident by the fact that iodide supplementation of the stock diet reduced these indices of thyroid function to values which are in the same range as those of rats. It is unlikely that the originally high values were caused by the presence of a goitrogen in the diet since the marked reductions in activity were brought about by the addition of only 1 to 2 μg of iodide per g of diet. Since the time when this work was done, Rogeler *et al.* (21) have published results of their findings on iodine requirements of the white Leghorn laying hen. They found that if hens were maintained with a presumably adequate iodine intake, their I^{131} uptake averaged 15%, whereas if hens had a very low iodine intake and then were maintained on the low-iodine diet, they had uptakes as high as 82%. Several groups of investigators have reported high thyroidal-I^{131} uptakes in birds (3,4); other investigators have reported much lower uptakes (1,2,5). Kobayashi *et al.* (3) found I^{131} uptakes of 61% in white-throated sparrows and values as high as 73% in weaver finches. The sparrows after capture were maintained in the laboratory on canary seed which contained 0.01 μg of iodine per g. The weaver-finch diet contained 0.03 μg of iodine per g and when this was supplemented so that the total iodine content was 0.13 μg

per g, no appreciable reduction in the peak-I^{131} uptake occurred. The investigators concluded that iodine supplementation of the diet had no effect on the thyroid activity in these birds. It would have been interesting to investigate if a larger iodine supplement would have reduced these high uptakes in the weaver finches considering that 0.13 μg of iodine per g of diet would represent a low supplement for rats or chickens.

It is proposed that the high residual activity persisting in the thyroid tissue of our chickens after hypophysectomy is attributable to an intense thyrotrophin stimulation caused by dietary-iodine insufficiency prior to removal of the pituitary. An attempt was made to measure levels of thyrotrophin in the plasma of these chickens by determining the potency of the chicken plasma in stimulating I^{131} uptake in hypophysectomized rats on low-iodine diet. However, the rats did not tolerate the chicken plasma very well; also, interpretation of the results of this test depends on the efficacy of chicken thyrotrophin in the rat. We have not yet tested this latter point.

There is still a possibility that the high residual thyroidal activity in the hypophysectomized chickens was the result of incomplete removal of the pituitary tissue. However, as was mentioned previously, when the pituitary sites of four hypophysectomized cockerels were examined in serial section no hypophyseal tissue could be found; these chickens had uptakes averaging 14% when they were sacrificed 5 months postoperatively. Also, as was shown in Table 6, I^{131} injected into the hypophysectomized chicken was distributed in the thyroid in a pattern markedly different from that in the intact bird; in the hypophysectomized chicken the percentage of thyroidal I^{131} that entered the thyroxine pool was only one-tenth of the corresponding percentage in the intact bird. This low percentage of label in thyroxine is characteristic of hypophysectomized rats as described by Taurog *et al.* (10), of hypophysectomized mice described by Wollman (16) and of hypophysectomized rats we have studied. It seemed of interest to examine the pattern of I^{131} in the thyroids of partially hypophysectomized rats to see whether the distribution would be characteristic of the normal or of the hypophysectomized rat, or lie somewhere in between the two. While our investigation of this problem has not been exhaustive, we found that in incompletely hypophysectomized rats the pattern of distribution of radioiodide in the thyroid was indistinguishable from that of the normal rat. However, the smallest remnant in any of the rats that were examined was approximately 1/32 of a pituitary gland.

In our chickens no significant reduction in average thyroid weight resulted from supplementation of the diet with iodine although rats fed the chicken stock diet for 6 months developed thyroids twice normal size. Nevertheless, quite often an individual chicken on stock diet had a highly enlarged thyroid gland. The circulating levels of thyroid hormone as indicated by serum-PBI

values were significantly increased by the iodine supplement but still remained low compared to levels found in rats (Table 4); they were also lower than those reported to date for chickens and other birds (22,23). We have not yet examined the chemical nature of the non-protein-bound-iodine fraction.

At the time the chickens were sacrificed (3 months of age), the thyroids of the intact and hypophysectomized birds on stock diet contained approximately the same amounts of iodine. This is striking in view of the marked reduction in thyroid weight in the hypophysectomized bird to almost one-third of normal. However, it is difficult to interpret this finding because the iodine contents of the birds at time of hypophysectomy, i.e. 6 weeks of age, were not determined. In this regard, it should be pointed out that with the stock diet the radioiodide uptake of the hypophysectomized cockerels per unit weight of thyroid tissue was as high as the corresponding value in the intact birds. An attempt will be made to compare the structures of the glands of the two kinds of chickens with respect to percentages of cells, colloid, and stroma and to correlate them with biochemical findings.

It is of interest to examine the iodine contents of the thyroid gland in relation to the amounts of hormone presumably secreted per day. In the case of intact rats on Diet I with average thyroidal iodine content of 11.5 μg and thyroxine-iodine content of 10% of total thyroidal iodine, i.e. 1.1 μg, the thyroxine content was 2 μg, or enough for 1 day's secretion by the gland (24,25). The total iodine content of the thyroid would be sufficient for 4 to 5 days' production of hormone. The intact birds on supplemented diet contained an average of 275 μg of iodine of which 11% was in the form of thyroxine, indicating a thyroxine-iodine content of 30 μg and hence a thyroxine content of approximately 50 μg. Assuming a thyroxine secretion rate in the bird of between 2 and 3 μg per day per 100 g of body weight (26,27), these cockerels which weighed approximately 1200 g should have secreted about 30 μg of thyroxine daily. Based on these figures, the chicken thyroids (supplemented diet) contained enough thyroxine for approximately one and a half days, and enough total iodine for a 9-day supply of hormone. A similar calculation from the data pertaining to chickens on stock diet reveal that the thyroid glands of these animals contained enough thyroxine for 1 day's secretion and enough total iodine for only 3 days' supply of hormone. In Paper II evidence is discussed indicating that the chickens on stock diet studied here secreted thyroxine at a much lower rate than is generally considered to be characteristic of chickens. In this regard, we wish to emphasize that "thyroxine-secretion rate" as usually defined in terms of the daily dosage of administered thyroxine, which is required to inhibit the release of previously concentrated I^{131} from the thyroid gland, probably overestimates the true rate of release of hormone from the gland. Under these conditions, it would seem that the pituitary is being completely inhibited with respect to release of thyrotrophin, a condition not characteristic of the

pituitary-thyroid relation when the thyroid is secreting its normal amount of thyroxine.

Concerning the distribution of iodine in the thyroglobulin of the cockerel, iodide (both I^{127} and I^{131}) appeared in the unhydrolyzed digests to the extent of only 0.5% and in the hydrolyzed digests to the extent of 2% of the total thyroidal iodine. Therefore the greater part of this 2% probably was formed during the hydrolysis by deiodination of organically bound iodine. However, this small amount of deiodination would not seriously have affected the observed distribution of iodine in the various components, even if the inorganic iodide had come all from the thyroxine pool which had the lowest abundance of total iodine. If the pattern of distribution of I^{127} in the intact cockerel on the iodine-supplemented diet is taken as a base-line, (Table 6) then the effect of low-iodine diet (stock diet) was to increase the relative abundance of thyroxine in the thyroglobulin, apparently at the expense of DIT; however, since the total thyroidal iodine decreased approximately threefold, there was less thyroxine in the iodoprotein of these birds on the stock diet despite the large increase in the relative abundance of the hormone. Hypophysectomy on the stock diet caused a reversion of the pattern of the iodinated amino acids toward that characteristic of the thyroid of the intact bird on the supplemented diet. However, removal of the pituitary on the supplemented diet did not significantly alter the iodine distribution except perhaps in thyroxine. The fact that the thyroglobulin of the chickens on stock diet contained a high percentage of thyroxine is consistent with the concept already proposed that the thyroids of the chickens on stock diet were highly stimulated by the pituitary gland because of the inadequate iodine content of the diet. When the birds were hypophysectomized, the stimulation was terminated but the glands retained a residuum of the pre-hypophysectomy activity; the reversion of the I^{127}-distribution pattern in these birds to that characteristic of the intact birds on the supplemented diet would coincide with the release of the thyroids from the excessive pituitary stimulation when the pituitary was removed. The iodinated-amino-acid-distribution pattern in normal cockerels reported by Vlijm (1) on the basis of I^{131} alone corresponds to the pattern of I^{127} in our intact birds on the iodine-deficient stock diet. However, the chickens described by Vlijm had peak I^{131} uptakes of only 10% to 23% which would suggest an adequate dietary iodine intake. No comparison will be made here with the distribution pattern for thyroidal I^{131} in birds reported by Kobayashi *et al.* (2,3) because these workers chromatographed butanol extracts of thyroids and provided no data for estimating the completeness of recovery of the amino acids from the extract. In hypophysectomized chickens the labelling of the thyroxine pool at 24 hr to the extent of only one-tenth of that in the intact bird might indicate a particular locus of action of thyrotrophin in the conversion of DIT to thyroxine. In hypophysectomized rats the quantity of I^{131} that failed to enter the thyroxine pool seemed to appear in the form of MIT. Since injected radioiodide does not

equilibrate with the iodine of the thyroxine pool in hypophysectomized animals as it does in intact animals and since iodine in the secreted thyroxine has a different specific activity in the two kinds of animals, it is meaningless to compare the rates of thyroxine secretion in the two groups on the basis of the rate of appearance of radioactive thyroxine in the circulation.

Seasonal variations in the I^{131} uptakes (3) and in the distribution of I^{131}-labelled amino acids in the iodoprotein of birds (28) have been reported. In our experiments with chickens over the past two and one-half years we have not seen any significant variation in the measured indices of thyroid functions which were attributable to the changing seasons. Perhaps this is because of the lack of extreme temperature variations in this locale.

In the thyroids of our rats on Diet I, the distribution of iodine (I^{127}) in the thyroglobulin was reasonably similar to that of I^{131} found at presumed equilibrium by Taurog et al. (10) and by Mayberry and Astwood (29). In our experiments with rats, as well as in those of Taurog and Mayberry and Astwood, equilibration of injected radioiodide seems to have occurred at 24 hr, except for the puzzling finding that the percentage of thyroidal I^{127} in the origin material on the chromatograms is twice as high as that of I^{131}. This anomaly has never been observed with chickens, and if further experiments in the rat should indicate that this is a real effect, it might be considered evidence for the existence of more than one metabolic pool of thyroidal iodoprotein in the rat.

In the case of the intact chickens, equilibration of injected radioiodide with the thyroidal components required approximately 48 hr (see Paper II). At this time interval after injection of I^{131} and at all later times (96 hr was the latest time at which the thyroids were examined) the relative distributions of I^{131} and I^{127} in the thyroglobulin were identical. This means that the relative specific radioactivity (percentage of thyroidal I^{131}/percentage of thyroidal I^{127}) of the iodine in all of the iodinated components reached the value of unity. Such a situation could occur if (1) all of the thyroglobulin behaves as though it were an iodine pool with a single metabolic-turnover rate, or if (2) the thyroglobulin comprises several pools of iodoprotein all with the same composition of iodinated amino acids in which iodine is metabolized at different rates. The present data do not allow a choice between the two possibilities.

SUMMARY

Various indices of thyroid function were studied in normal and hypophysectomized, 3-month-old white Leghorn cockerels maintained on a low-iodine stock diet and on the stock diet supplemented with iodide. The indices studied were I^{131} uptake, T/S ratio, I^{127} content of the thyroid, and serum-I^{127} levels, both protein-bound and total iodine. The distribution of iodine (both I^{127} and I^{131})

in the various iodinated components of the thyroidal iodoprotein was determined after fractionation by paper chromatography, and the effect of iodine content of the diet and of hypophysectomy investigated. Some experiments were performed in rats as a basis of comparison. The studies revealed a low level of circulating thyroid hormone in the serum of intact chickens on the stock diet and a hyper-activity of the thyroid gland; supplementation of the diet with iodide strikingly reduced this activity. Thyroid activity of rats maintained on this low-iodine diet was comparably high as was that in the chickens. When the cockerels were hypophysectomized, although the target organs atrophied and the thyroid activity decreased, as measured by the stated indices of function, an unusually high residuum of activity remained in the thyroid glands. Nevertheless, following the injection of I^{131} the defect, characteristic of hypophysectomized rats and mice, was observed in the labelling of thyroidal thyroxine. Similarly, the thyroids of hypophysectomized rats maintained on low-iodine diet retained high activity if they were from mothers also maintained with inadequate iodine. It is postulated that the unusually high residual activity persisting after hypophysectomy in the thyroid tissue of the chickens and rats on low-iodide diet is attributable to an intense thyrotrophin stimulation caused by dietary iodine insufficiency prior to the removal of the pituitary.

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Thyroid Function in Chicken and Rat. II. The Equilibration of Injected Iodide with Existing Thyroidal Iodine in Chicken

Lawson L. Rosenberg, Gilles La Roche and Mildred K. Dimick

Paper I of this series reported on certain aspects of thyroid function in white Leghorn cockerels from hens on a stock diet with very low iodine content (0.03 μg iodine per mg) and themselves maintained on low-iodine diet. The thyroid glands of these birds were extremely hyperplastic; the average I^{131} uptake was 50% of the injected radioiodide even when the isotope was administered with 50 μg of carrier I^{127}. The average T/S ratio with "blocked" thyroids was more than 600, the circulating-PBI level was only 0.5 μg per 100 ml of serum, and total circulating iodine was 0.7 μg per 100 ml. When an iodide supplement was added to the diet, these indices of thyroid function were altered dramatically; the radioiodide uptake was reduced threefold, T/S ratios were reduced fivefold, and the PBI level was increased by 60% of its original value. When the chickens on stock diet were hypophysectomized, their thyroids retained a considerable degree of activity 6 weeks postoperatively as judged by I^{131} uptake and T/S ratio.

Supplementation of the diet with iodine caused a threefold increase in the iodine content of the thyroids of the cockerels and produced a profound change in the distribution of iodinated (I^{127}) amino acids in the iodoprotein. The relative abundance of thyroxine in the iodoprotein decreased to half of its corresponding value in the animals on stock diet and that of DIT increased markedly.

The effects of hypophysectomy and of the amount of dietary iodine on the distribution of I^{131} 24 hr after its injection were described in Paper I and compared with the distribution of I^{127} in the existing thyroidal-iodine stores. The present paper describes the kinetics of equilibration of iodine newly taken into the thyroid (I^{131}) with iodine already present (I^{127}) in intact cockerels on stock diet. The time course of the labelling is consistent with the concept that iodination and deiodination reactions occur continually in the gland of intact animals and lead to complete randomization of all thyroidal iodine. If this concept proves correct, the rate of net synthesis of thyroxine in the thyroid can obviously not easily be estimated from the rate at which the hormone becomes labelled in the thyroglobulin after injection of radioiodide.

MATERIALS AND METHODS

White Leghorn cockerels were used at 3 months of age. The cockerels were hatched from hens on a stock diet which contained 0.03 μg of iodine per g of diet and were thereafter maintained on a diet with an iodine content of 0.14 μg per g. They were injected intravenously with carrier-free I^{131} (NaI^{131} from Oak Ridge) and were sacrificed after various intervals in groups of five. The thyroid gland of each chicken was removed and homogenized in Tris-NaCl buffer.

An aliquot was taken to calculate uptake prior to digestion proceeding at 37°C for 24 hr in the presence of added pancreatin. The iodinated components of the digest mixtures were fractionated by ascending chromatography in collidine-ammonia solvent. The radioactive bands, localized with autoradiographs, were cut from the chromatograms and counted in a well-type scintillation counter. Chemical iodine (I^{127}) analyses were then performed directly on the same bands. Three chromatograms of each thyroid digest were made, two for determination of both I^{131} and I^{127} and one with added markers to identify labelled amino acids. The exact procedure is described in detail in Paper I.

RESULTS

1. Thyroidal Content of I^{131} as a Function of Time after Injection of Radioiodide. Fig. 1 shows the percentages of the injected radioactivity present in the thyroid at various time intervals after injection of I^{131}. Concentration of the I^{131} appeared to have been essentially complete after about 6 hr. The peak uptake was approximately 32% of the injected dosage, which was lower than the 50% uptakes observed previously (Paper I) in similar chickens on the stock diet. A striking feature of these observations is that the I^{131} content of the glands did not diminish significantly in the four-day period following the initial uptake of the I^{131} even though on the second day the level of I^{131} in the blood had fallen to only 0.004% of the injected dosage per ml.

2. Distribution of I^{131} in the Components of the Hydrolyzed Thyroidal Iodoprotein. Fig. 2 shows the distribution pattern of I^{131} in the pancreatin hydrolysates of thyroids at various time intervals after injection of radioiodide. It is apparent that the label had quickly entered the MIT and DIT and then, more slowly the thyroxine fraction. The percentage of thyroidal I^{131} in the MIT of the hydrolysates was 48% at the earliest time, decreased as time progressed and at approximately 48 hr approached an equilibrium value of 28%. The relative abundance of I^{131} in DIT remained almost constant at 39% from the earliest time to the latest, and in thyroxine increased from 2% at one and a half hours to an equilibrium value of 22% of the thyroidal I^{131} at about 48 hr. The increase in relative abundance of the label with time in the thyroxine fraction appeared to be at the expense of that in MIT. The amount of thyroidal I^{131} in the inorganic-iodide compartment remained constant at approximately 2%, and the

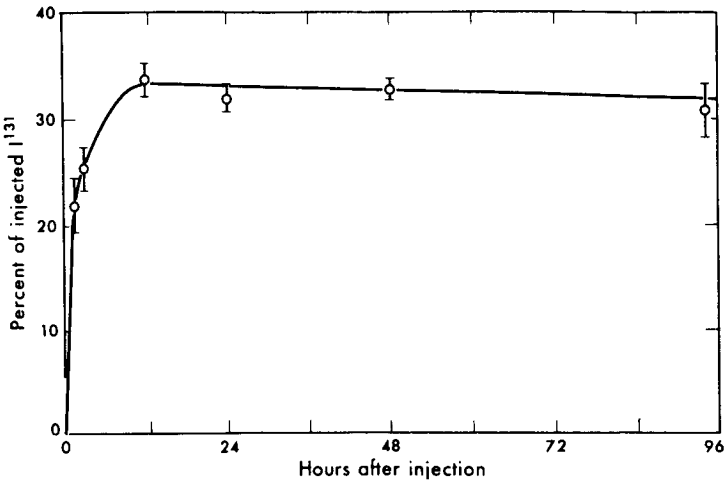

Figure 1. I^{131} uptake curve. MU-29978

amount remaining at the origin of the chromatograms was 8%. No triiodothyronine has ever been detected in digests of thyroids of these chickens. In Paper I we have presented evidence that the material at the origin was undigested thyroglobulin whose composition was identical with that which had been hydrolyzed; we have also presented evidence that the distribution of I^{131} in the amino acids of the hydrolysate truly represented the distribution of radioiodine in the amino acids of the iodoprotein of the intact thyroids and was not an artifact of the hydrolysis procedure. Recoveries of I^{131} from a given aliquot of thyroid-digest mixture were quantitative after fractionation by paper chromatography.

3. Distribution of I^{127} in the Components of the Hydrolyzed Thyroidal Iodoprotein. Table 1 shows the distribution of I^{127} in the components of the digests of the chicken thyroids. Since each molecule of DIT contains 2 atoms of iodine and each of thyroxine contains 4 atoms, this distribution corresponds to abundances of MIT, DIT, and thyroxine in the iodoprotein in the molar ratios 10:7:2, respectively. I^{127} determinations were made on duplicate chromatograms of one digest at each time interval; these data are given in full in Table 1 to illustrate the extent of recovery in such experiments after fractionation of the components by chromatography. Pickering *et al.* (1) reported similar quantitative recoveries of I^{127} from chromatograms of digests of thyroids of foetal monkeys.

4. Relative Specific Activities of Iodine in the Components of the Hydrolyzed Thyroidal Iodoprotein. In Fig. 3 the results are plotted in terms of "relative" specific activity, which we define as the percentage of thyroidal I^{131} in a particular component divided by the percentage of thyroidal I^{127} in the same component. The figure shows clearly that the relative specific activities of the iodine in the thyroidal components all reached the value of unity at approximately 48 hr.

Table 1. Distribution of I^{127} in thyroidal components as determined on paper chromatograms

Time after injection I^{131} hr	Iodine present in chromatograms* of 40 µl thyroid digest as the following components						Iodine in 40 µl thyroid digest µg	Recovery of iodine on chromatograms %	Total thyroidal I^{127}† µg
	Origin	MIT	DIT µg	T_4	Iodide	Total			
1-1/2	0.254	1.057	1.382	0.915	0.064	3.672	3.95	93	95 ± 15‡
	0.217	0.984	1.264	0.860	0.070	3.395		86	
	6.6%	28.9%	37.5%	25.1%	1.9%				
3	0.306	0.922	1.359	0.752	0.053	3.392	3.93	86	94 ± 8
	0.332	1.060	1.500	0.909	0.046	3.847		98	
	8.8%	27.4%	39.6%	22.9%	1.4%				
12	0.280	0.821	0.992	0.545	0.024	2.662	2.69	99	81 ± 8
	0.307	0.718	1.121	0.641	0.039	2.826		105	
	10.7%	28.1%	38.5%	21.6%	1.2%				
24	0.245	0.748	1.025	0.568	0.021	2.607	2.53	103	85 ± 9
	0.262	0.768	1.069	0.604	0.036	2.739		108	
	9.6%	28.4%	39.2%	21.9%	1.0%				
48	0.202	0.498	0.750	0.397	0.041	1.888	2.12	89	86 ± 17
	0.224	0.551	0.805	0.446	0.032	2.058		97	
	10.8%	26.6%	39.4%	21.4%	1.9%				
96	0.292	0.819	1.360	0.750	0.066	3.287	4.00	82	92 ± 14
	0.316	0.848	1.367	0.772	0.066	3.419		85	
	9.0%	25.6%	40.7%	22.7%	2.0%				
avg	9.2%	27.5%	39.1%	22.6%	1.6%				

*The two chromatograms at each time interval are duplicate chromatograms of a single thyroid digest.

†Average of 5 animals per group.

‡Standard error of the mean.

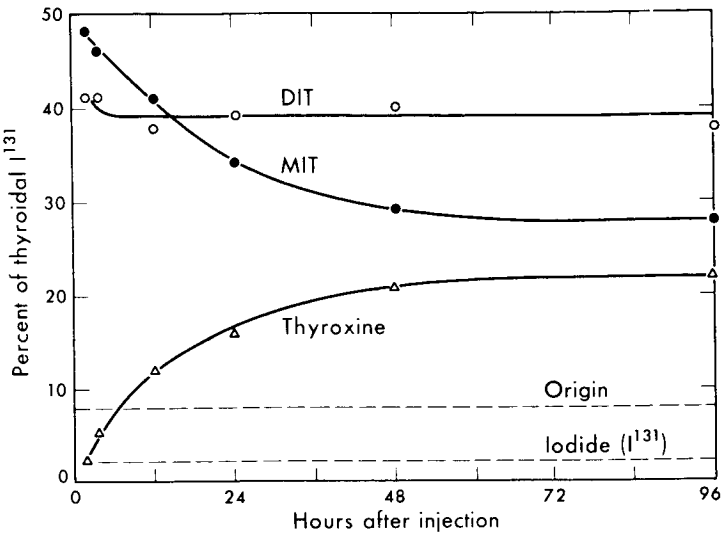

Figure 2. Distribution of I^{131} in thyroid metabolites. MU-29977

DISCUSSION

Four days after injection of radioiodide when the I^{131} in the plasma was at a very low level (I^{131} in whole blood had already fallen to 0.004% of the injected dosage by the second day) the thyroid glands still contained essentially all of the radioactivity they had concentrated at the early time of maximum uptake (Fig. 1). The long-time retention of thyroidal I^{131} indicated a low thyroxine-secretion rate for these chickens. Pipes et al. (2) described chickens with high uptakes whose thyroids retained radioiodide in a similar manner. Kobayashi et al. (3) called attention to long retention of I^{131} in thyroids of birds. However, in 7-to-10-week-old cockerels, Vlijm (4) found a half-time of I^{131} in the thyroid of about 2 days. In rats, values of 5 days (5) and 3 days (6) have been reported.

For reasons cited earlier in this paper and in Paper I, we believe that the thyroid glands of the chickens in the present experiments are not characteristic of chickens per se but rather of animals maintained chronically with inadequate dietary-iodine intake, and that their high uptakes and high T/S ratios, low circulating-thyroxine levels (PBI) and slow release of radioactivity reflect the low availability of iodine for the synthesis and secretion of adequate amounts of thyroxine. In this regard it is interesting that many physiological functions of animals can be maintained adequately by the daily injection of much smaller amounts of thyroxine than those believed to be secreted daily by the thyroid gland (7).

If the progress of iodine through the thyroid gland is the result only of iodination of hormone precursors followed by synthesis and release of hormones, then the labelling of the thyroidal components in the thyroglobulin following injection of I^{131} should occur as follows, providing that the relative distributions of iodinated (I^{127}) components in the thyroid remain constant and

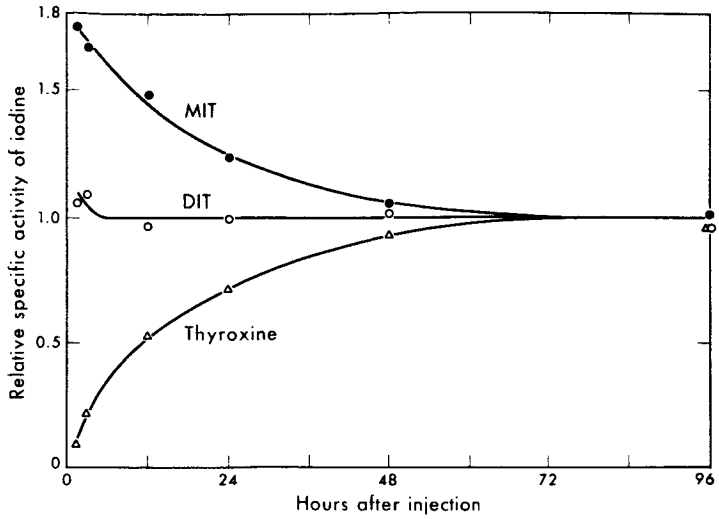

Figure 3. Relative specific activities of iodine in thyroidal metabolites.
MU-29976

that no special small, highly metabolically active pool of iodoprotein exists:

1. Radioiodide should appear early in MIT and DIT at the same rate since, presumably, both are being labelled from the same iodide pool by iodination of pre-existing tyrosine and MIT (unlabelled) in the thyroglobulin.
2. Since MIT and DIT at early times should be labelled at the same rate, their specific activities at those early times will be determined by their concentrations in the thyroglobulin.
3. As the concentration of radioiodide in the blood decreases with time following injection of I^{131}, the specific activity of the labelling iodide will decrease and the MIT fraction will lose radioiodine. This is because the MIT that is leaving the pool to form DIT was synthesized earlier when the specific activity of the labelling iodide was high; this MIT therefore has a higher specific activity than that being newly formed to replace it.
4. MIT and DIT can become highly labelled even with the movement of small quantities of iodine through the gland since the labelling iodide has a very high specific activity, i.e., a large amount of radioactivity is associated with a very small quantity of iodine.
5. Thyroxine should become labelled less quickly than DIT if thyroxine is synthesized from DIT and if the label in DIT is diluted uniformly in the total DIT pool. This must be true especially in the thyroids of the chickens used in the present experiment because DIT comprises the largest iodine pool. For thyroxine to become substantially labelled, a significant part of the iodine of the DIT pool, and therefore a significant part of the total thyroidal iodine, must be moved into the thyroxine pool. Such a movement would have to be accompanied by the secretion of an equivalent amount of iodine from the gland as thyroxine; otherwise the thyroxine content of the thyroglobulin would increase continually at the expense of precursor components.
6. On the other hand, if thyroxine is formed by iodination of thyronines, the label should appear initially in thyroxine at a rate comparable to that of its appearance in MIT and DIT.

It should be stated immediately that our data concerning the equilibration of newly acquired iodine (I^{131}) with existing thyroidal iodine (I^{127}) do not conform to the above theoretical considerations. In order to explain our findings we have proposed a different pathway for the movement of iodine within the gland.

In our experiments the relative amounts of MIT, DIT, and thyroxine (based on I^{127}) did not change during the 4-day course of the experiment; therefore, no significantly large quantity of newly synthesized thyroxine could have accumulated in thyroid unless during that time interval the same amount of previously formed thyroxine was released from the glands, or unless a simultaneous proportionate increase in MIT and DIT occurred. As mentioned earlier, the long retention of I^{131} in the thyroids of these cockerels, in addition to the low levels of circulating thyroxine (PBI) and the other indices of thyroid function suggest that thyroxine was released only slowly from the gland. Of course, it is recognized that the level of circulating hormone does not necessarily reflect the rate of secretion of hormone from the gland; rather, it is the resultant of the rate of secretion and of its peripheral utilization.

Pipes et al. (2), who reported similar long-time retention of I^{131} in the thyroids of chickens, assumed that the failure of the thyroids to release the radioactivity was only apparent, and that although they secreted thyroxine at a presumably normal rate, the glands recovered the I^{131} from the peripheral radioactive thyroxine with very high efficiency. In support of this argument they showed that propylthiouracil caused a rapid release of previously concentrated thyroidal radioiodine, from which they concluded that the drug had prevented re-utilization of the I^{131} of radioactive thyroxine previously secreted from the gland. It seems unlikely that the thyroid glands of our cockerels, which concentrated only approximately 33% of injected radioiodide and allowed the remainder to escape, could then with very high efficiency recapture the radioiodine from secreted thyroxine, unless the iodide released by peripheral utilization was carried in a special form for which the thyroid had special affinity.

In view of the arguments presented above, if movement of iodine within the thyroid gland of the chickens had resulted only from iodination of precursors followed by synthesis and release of hormone then MIT and DIT should have become quickly labelled. However, thyroxine should not have become labelled significantly during the course of the experiment because only a very small percentage of the total thyroidal iodine would have left the gland during that time. Examination of the results presented in Fig. 2, show that labelled MIT and DIT did indeed appear quickly in the iodoprotein, but they also show that the thyroxine pool became significantly labelled during the experiment, although at a slower rate than MIT and DIT. An examination of the manner in which I^{131}

equilibrated with thyroidal stores of iodine (I^{127}) reveals that the specific activities of the iodine in the components which were separated on the chromatogram all had become identical and equal to unity after 48 hr (Fig. 3).

We suggest that this randomization of all of the thyroidal iodine was accomplished by the occurrence in the thyroid of continual iodination and deiodination reactions in such a way that the iodine is continually recirculated intrathyroidally. We suggest further, on the basis of published results of other workers to be discussed below, that this randomization of thyroidal iodine is not unique in these chickens with inadequate iodine intake but rather is characteristic of chickens and other species as well. If the iodinated amino-acid residues are deiodinated when they are in peptide linkage in the thyroglobulin, it is at least conceivable that the deiodinations merely represent the reversal, at the enzyme surfaces, of the iodination reactions. However, this reversibility might seem unlikely in view of the probable nature of the biological oxidation of iodide and of the iodination reaction (8). If the iodinated amino acids are deiodinated when they are in free state, then this reversible mechanism for iodination and deiodination would be even less likely in view of the accepted notion that the amino-acid residues are iodinated while they are in peptide linkage in the thyroglobulin.

Roche et al. (9) early suggested that the deiodinase present in thyroid tissue had the function of conserving the iodine of the hormone precursors which are presumably released from peptide linkage during proteolysis of the thyroglobulin. Their results indicated that the deiodinase had no action on the iodinated thyronines, and acted on iodinated tyrosines only when they were present as the free amino acids and not when they were in peptide linkage. However, recently Pitt-Rivers and Tata (10) have found that a deiodinase from pig thyroid gland deiodinated thyroxine, if it was free of blood proteins.

Our data do not provide information to allow a choice among the various possible ways in which continual iodinations and deiodinations can lead to randomization of the thyroidal iodine. We interpret our results on the equilibration of injected I^{131} with existing thyroidal I^{127} in the following way. At the earliest times, the pattern of labelling with I^{131} should be the result, predominantly, of the initial iodination reactions. Under these conditions the specific activity of MIT should be twice that of DIT since MIT has one iodine atom and DIT has two. At one and a half hours, the earliest time the thyroids were examined in this experiment, the specific activity of MIT was 1.75 times that of DIT. An explanation for the finding that the specific activity of DIT was equal approximately to unity already at one and a half hours is not apparent. It will be interesting to examine the distribution of iodine at times earlier than this. As the specific activity of the labelling iodine decreases with time, the effect of the succeeding deiodinations and reiodinations would become dominant

in determining the labelling of the iodinated compartments and should lead to randomization of the I^{131}. It is suggested here that even if no net synthesis of the hormone occurs, thyroxine of the thyroglobulin can equilibrate with the total thyroidal iodine, whether or not the thyroxine can be deiodinated directly; if thyroxine is in reversible equilibrium with its presumed precursor, DIT, then the continual iodination and deiodination of DIT would effectively bring about equilibration of thyroxine iodine.

Roche et al. (9) had found that thyrotrophin increased the activity of the thyroidal deiodinase. I. N. Rosenberg et al. (11) demonstrated that in dogs whose thyroids had been labelled with I^{131}, subsequent injection of thyrotrophin caused secretion of iodide, both I^{127} and I^{131}, into the venous blood, and in the rat and in man (12), caused an increase in the intrathyroidal concentration of iodide. They speculated that this iodide arose from deiodination of thyroidal organic components. Wollman and Scow (13) and Wollman (14) considered the possibility of the existence of two separate iodide pools in the thyroids of mice and rats in order to explain the increased T/S ratios with time after injection of I^{131} when organic binding of iodine was allowed. For the same reason, Halmi and Pitt-Rivers (15) recently postulated the existence of an iodide pool arising from deiodination of iodotyrosines and distinguishable from that of iodide originally transported into the gland in that the retention of the latter is affected by perchlorate. They suggested that some of the iodide in the second pool is recirculated into the blood via the "transport mechanism," but that most of it is recycled within the thyroid by direct incorporation into hormone precursors. These suggestions are consistent with our findings.

Mayberry and Astwood (5) reported that the relative distribution of I^{131} in the thyroid of rats remained constant over a 7-day period following the injection of I^{131} even though the total radioactivity in the gland during that period decreased to 40% of its 24-hr-content. These findings are also consistent with the hypothesis of randomization of thyroidal iodine, as would be expected with the occurrence of continual iodination and deiodination.

If it is true, as we believe, that our chickens on stock diet retained their thyroidal radioiodide because they secreted thyroxine only slowly, and if randomization of thyroidal iodine is a general phenomenon and is not limited to these particular chickens, then these are suitable experimental animals in which to investigate the mechanisms for intrathyroidal interconversions of iodine. The fact that in our experiments with these chickens the thyroxine of the thyroglobulin, although synthesized at a very low rate, became highly labelled with injected I^{131}, and that the relative specific activities of the iodine of all of the iodinated components attained the value of unity has led us to consider that intrathyroidal deiodinations are important in the metabolism of

the thyroid gland. In addition, if these concepts are verified, then calculations concerning the rate of net synthesis of thyroxine based on the rate of appearance of injected label in the thyroidal-thyroxine fraction have no meaning, since thyroxine can become labelled without any net synthesis. Further, the findings of Pipes et al. (2) that propylthiouracil caused a rapid release of previously concentrated thyroidal I^{131} from thyroids of chickens, which under ordinary circumstances retained the radioiodine, could be explained by the interference of the drug with continual deiodinations and iodinations. This possibility has already been suggested by Slingerland et al. (16) to explain their findings in experiments with normal human subjects. Thus, the iodide freed by the deiodinations might be lost from the gland. Experiments are in progress to test these ideas further in both chickens and rats.

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Differentiation between Vitamin-B_{12}- and Folic-Acid-Deficient Megaloblastic Anemias with C^{14}-Histidine

Mathews B. Fish, Myron Pollycove and Thomas V. Feichtmeir

Megaloblastic anemias result from a relative or absolute deficiency of vitamin B_{12} or folic acid. Often it is difficult to make this etiologic differentiation, either from clinical observations or by use of routine hematologic procedures. Several methods, which have been devised to evaluate folic acid deficiency, have been reviewed recently (1). These include: therapeutic trial of "physiologic doses" of folic acid (2); determination of serum-folic-acid activity (3,4); determination of rate of clearance from the plasma of intravenously administered folic acid, and determination of amount of folic acid in blood and urine after oral or parenteral doses of folic acid (5,6,7); and the urinary formiminoglutamic acid (FIGlu) determination after large oral doses of histidine (8,9,10,11,12,13,14,15). Some of these methods are helpful in the differentiation of folic-acid and vitamin-B_{12} deficiency in megaloblastic anemias, but overlapping results have been noted frequently. As these approaches are primarily diagnostic in nature, the results obtained add little information regarding the basic metabolic abnormalities in folic-acid or vitamin-B_{12} deficiency. The determination of urinary FIGlu serves as an index of the folic-acid-dependent conversion of formiminoglutamic acid to glutamic acid. However, the large oral dose of histidine administered in this test is unphysiologic and may be responsible for the overlapping results obtained in folic-acid deficiency, vitamin-B_{12} deficiency (2,3,16) and other hematologic and nonhematologic states (17,18,19).

It is known that folic acid is specifically concerned with the metabolic reaction involving the transfer and utilization of single carbon moieties (20, 21). Recent studies suggest that vitamin B_{12} is also required in certain aspects of this metabolic process; it may be essential in methionine-methyl neogenesis (20).

Histidine is an important contributor to the single-carbon-moiety pool (22). Some aspects of the metabolism of C^{14}-labeled histidine (specifically L-histidine-2(ring)-C^{14}, the 2-carbon atom being contributed to the single-carbon-moiety pool) were studied in the mouse (23), rat, monkey, and human (24, 25,26). These studies demonstrated the very rapid incorporation of radioactivity into visceral protein and rapid appearance of $C^{14}O_2$ in the expired air (23), the specific activity of various urinary metabolites of C^{14}-histidine and the

$C^{14}O_2$-specific activity in the expired air of rats (24,25,26).

The present study, using C^{14}-histidine, is an attempt to differentiate between folic-acid and vitamin-B_{12} deficiency and to investigate the pathologic physiology in patients with megaloblastic anemia.

MATERIALS AND METHODS

Blood and bone-marrow studies were performed by standard methods (27). The diagnoses of megaloblastic anemia were made by the characteristic blood and marrow findings. Bone-marrow-hemosiderin samples were prepared (28) and graded 0 to 4+ on the basis of the number of hemosiderin granules observed. All patients with a megaloblastic bone marrow also demonstrated the uniformity of hemosiderin distribution characteristic of intramedullary hemolysis (28). Vitamin-B_{12} absorption was measured by either the Schilling test (29) or hepatic-uptake test (30).

The diagnosis of the specific vitamin deficiency was eventually confirmed by the characteristic reticulocyte response to "physiologic doses" of the corresponding vitamin (2) and/or vitamin-B_{12}-absorption studies in patients who had been on folic acid-"free" diets (0.2-0.4 mg/day) (Table 1). Patient M.G. received a regular diet (folic acid 1-2 mg/day) (31,32,33), and his reticulocyte count rose from 1.1% at time of initial examination to 2.3% at the time of C^{14}-histidine study, reaching a peak of 10.9% on the 9th day after admission. Patient W.C. developed a reticulocyte increase from 0.9% (Hb concentration 10.7 g/100 ml) to 7.9% 5 days after parenteral injection of 1000 μg of vitamin B_{12} (Schilling test). Patient G.M. had acute lymphatic leukemia and developed megaloblastic anemia after receiving amethopterin 5 mg daily, for 3 months. When amethopterin therapy was discontinued after the initial C^{14}-histidine study, a spontaneous reticulocyte increase occurred with a peak of 32% on the 5th day.

Expired-$C^{14}O_2$ specific activity was determined continuously on the breath exhausted from a spherical plastic helmet and passed through an infrared CO_2 analyzer and a 22-liter ionization chamber with vibrating-reed electrometer similar to that described previously (34). After baseline measurements were obtained for 20-30 min, all subjects tested received an intravenous injection of approximately 25 μg of L-histidine-2(ring)-C^{14},* specific activity 7.6 mC/millimole, radiochemical purity 97-99%, and breath measurements were continued for 2-4 hours. During this procedure serial blood and urine samples were obtained for the determination of serum-folic-acid and vitamin-B_{12} activity, total radioactivity and amino-acid specific activities. Results of these blood and urine analyses will be presented elsewhere.

*Obtained from Nuclear-Chicago Corporation, Des Plaines, Illinois.

Table 1. Hematologic laboratory findings at time of initial examination of eight patients with megaloblastic anemia and two normal subjects

Diagnosis	Subject	Age	Sex	Wt kg	Hb g/100 ml (12-18)	Hct % (37-54)	RBC $10^6/mm^3$ (4.2-6.2)	MCV μ^3 (82-92)	MCH $\mu\mu g$ (27-31)	MCHC % (32-36)	Reticulocytes % (0.5-1.5)	WBC $10^3/mm^3$ (4-12)	Reticulocyte response to physiological dose vitamin: Vitamin	Dose/day	Retic.	Day	Vitamin-B_{12} absorption: Test	$\bar{s}$ IF	$\bar{c}$ IF	Notes
Normal	Control 1	27	M	82	----	48	5.20	90	--	34	---	---	---	----	---	--	----	----	----	----
Normal	Control 2	41	M	83	14.0	41	5.10	81	28	34	0.8	6.2	---	----	---	--	----	----	----	----
Vitamin-B_{12} deficiency	M.F.	73	M	55	9.0	24	2.09	116	43	37	0.9	4.0	B_{12}	1 µg	16%	5	GI absorption*	1.9%	18.5%	1, 4
Vitamin-B_{12} deficiency	W.C.	44	M	53	10.7	33	2.77	119	39	33	0.9	9.5	---	----	---	--	Urinary excretion†	0.7%	----	2, 5
Vitamin-B_{12} deficiency	J.B.	84	M	59	4.2	14	1.08	127	38	30	0.1	2.2	B_{12}	1 µg	20%	10	GI absorption*	9.2%	51.3%	1
Vitamin-B_{12} deficiency	C.K.	86	M	50	11.7	33	3.30	101	36	35	1.6	7.0	B_{12}	1 µg	7%	6	Urinary excretion†	0.5%	3.8%	1, 6
Folic-acid deficiency	R.M.	30	F	55	4.5	14.5	1.50	98	30	31	0.3	2.9	Folic acid	0.5 mg	24%	5	----	----	----	1, 7
Folic-acid deficiency	F.M.	25	F	90	3.9	13	1.43	92	28	30	1.0	6.1	---	----	---	--	----	----	----	1, 8
Folic-acid deficiency	M.G.	59	M	61	6.5	19.8	1.64	121	40	33	1.1	2.4	---	----	---	--	----	----	----	2, 9
Folic-acid block	G.M.	5	M	23	5.8	16.5	1.73	95	34	35	1.0	6.0	Endogenous folic acid	----	32%	5	----	----	----	3

*Hepatic-uptake test (30).
†Schilling test (29).

Notes

1. Hematologic status at time of initial C^{14}-histidine study essentially unchanged from that found at time of initial examination.
2. Patient responding to vitamin therapy at time of initial C^{14}-histidine study.
3. Patient G.M. developed his anemia after a complete clinical and hematologic remission was obtained while receiving amethopterin, 5 mg/day for 3 months.
4. Combined system disease.
5. Partial terminal small bowl resection and active regional enteritis of most of remaining small bowl.
6. Pneumococcal pneumonia during period of expected maximal reticulocyte response.
7. Known chronic alcoholic with poor dietary intake.
8. Pregnant--32-35th week. Poor dietary intake.
9. Chronic alcoholic with poor dietary intake.

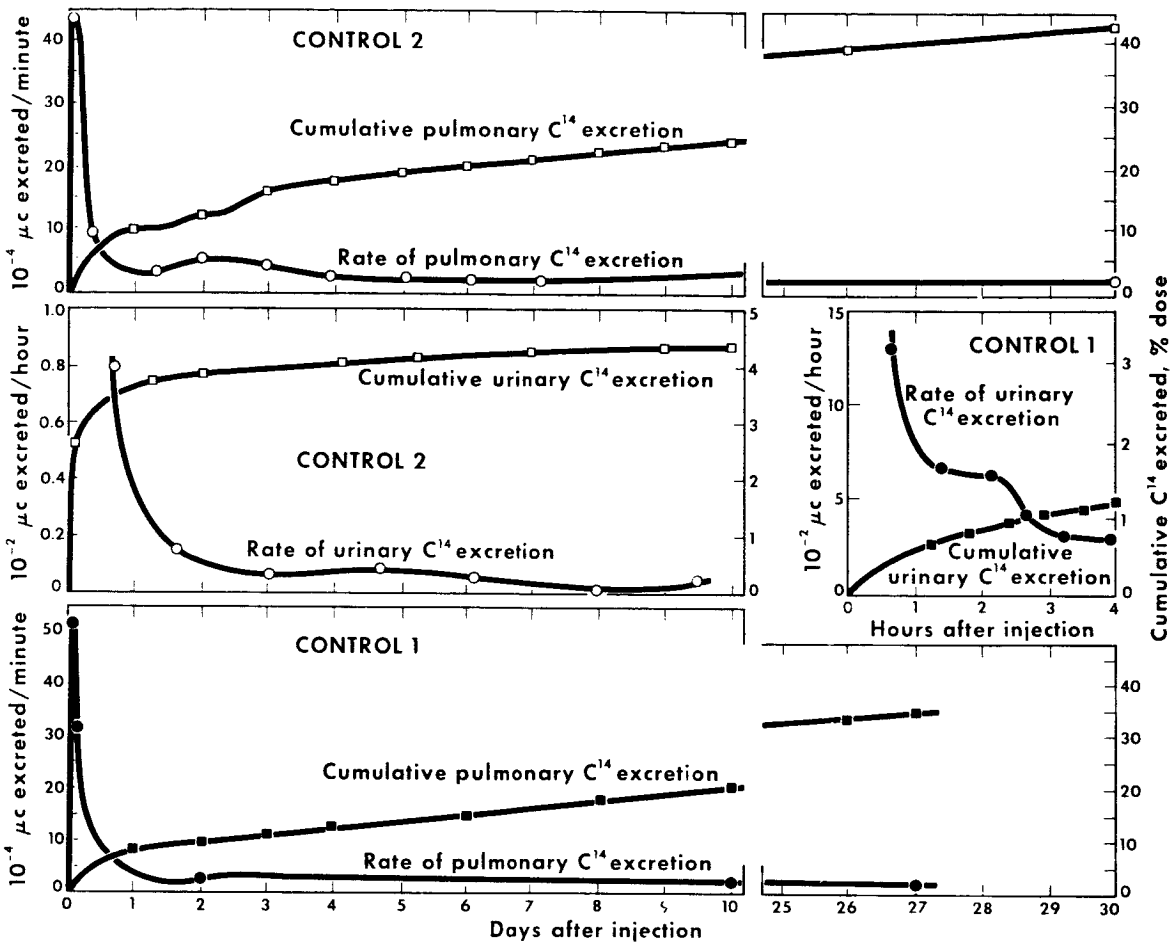

Figure 1. Pulmonary and urinary excretion of C^{14} by two normal subjects after intravenous administration of 0.5 mg L-histidine-2(ring)-C^{14} (approximately 25 μC).
MUB-1234

RESULTS

The cumulative excretion of C^{14} in breath and urine of two normal subjects is presented in Fig. 1. Breath-analyses parameters and hematologic findings in two normal subjects and in eight patients with megaloblastic anemia are presented in Table 2. Some of the patients were studied initially during relapse, a second time during a period of reticulocytosis in response to vitamin therapy and a third time during remission after the subsidence of reticulocytosis (Fig. 3, 4). Others were studied during one or two of these phases.

The $C^{14}O_2$-specific-activity curves of the expired air obtained in a normal subject, in a patient with vitamin-B_{12} deficiency in relapse and in a patient with folic-acid deficiency are shown in Fig. 2. The specific activity is expressed in microcuries per gram of carbon of expired CO_2 per 10 microcuries of C^{14} injected. In the normal subject (Control 1) the specific activity rises rapidly and reaches a maximum at approximately 40 min after injection (T_{max}=40 min)

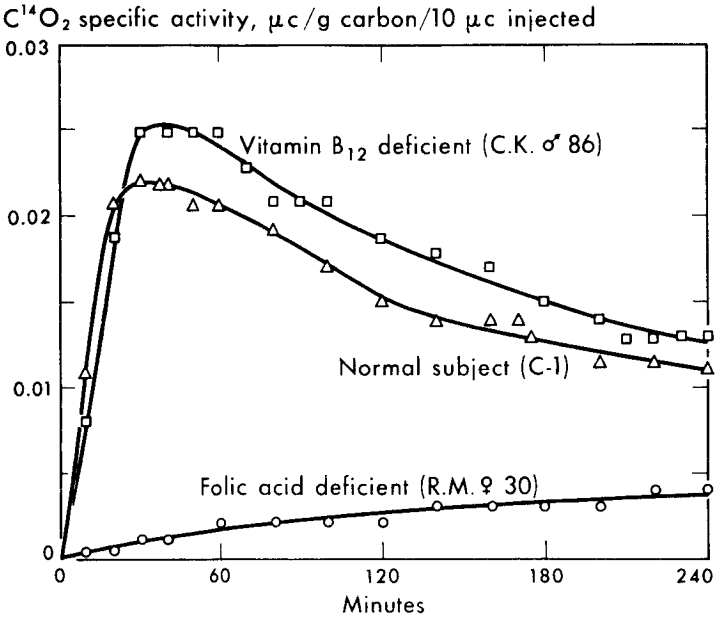

Figure 2. $C^{14}O_2$ specific activity in a normal subject, a patient with vitamin-B_{12}-deficiency megaloblastic anemia and a patient with folic-acid-deficiency megaloblastic anemia after intravenous administration of 0.5 mg L-histidine-2(ring)-C^{14} (approximately 25 μC).

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and then gradually declines so that the cumulative 1-hour excretion of C^{14} in the breath totals 1.18% of the injected C^{14}.

In the vitamin-B_{12}-deficient patient (C.K.) the specific-activity curve is of the same magnitude and slope characteristics as noted in the normal subject, reaching its maximum at 40 min, and in one hour 0.73% of the injected activity appears in the breath. By contrast, in the folic-acid-deficient patient (R.M.) the specific-activity curve rises slowly, reaching a relatively low and delayed maximum at 4 hours, and only 0.06% of the injected activity appears in the breath in one hour.

The breath-$C^{14}O_2$-specific-activity curves in the vitamin-B_{12}-deficient patient (M.F.) during relapse, response and remission are shown in Fig. 3. The graphic insert shows the time relationship of each study to the patient's hematologic course. Prior to the treatment, on day zero, the specific-activity curve is of the same magnitude and character as previously shown in the normal subject and in the vitamin-B_{12}-deficient patient (Fig. 2). On day 6, at a time of increased effective erythropoiesis as evidenced by the marked reticulocytosis, the specific-activity curve is quantitatively much lower than on day zero, yet the shape is similar in that a maximum is reached at 28 min followed by a gradual decrease. Finally on the 36th day, when the patient was in hematologic remission, the specific-activity curve is again of the same magnitude and shape as noted in the normal subject and initially in this vitamin-B_{12}-deficient patient.

These differences and changes noted--in the normal subject, in the vitamin-B_{12}-deficient patient and in the folic-acid-deficient patient--during relapse,

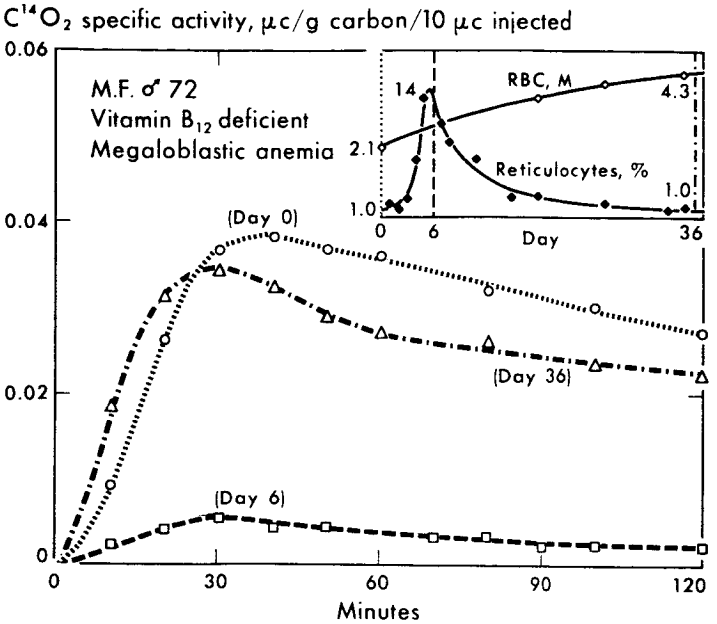

Figure 3. $C^{14}O_2$ specific activity in a patient with vitamin-B_{12}-deficiency megaloblastic anemia occurring after intravenous administration of C^{14}-histidine during relapse (Day 0....), reticulocyte response (Day 6----), and remission (Day 36-.-.-.-.).
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response and remission were found consistently in all patients studied. Normal subjects, vitamin-B_{12}-deficient patients in relapse, and patients in remission demonstrated similar T_{max} and cumulative 1-hour C^{14} breath excretion: the T_{max} occurred within an hour (20-50 min) and the cumulative 1-hour C^{14} breath excretion was approximately 1% (0.71%-1.34%) (Table 2). Patients studied during a reticulocyte response to vitamin-B_{12} or folic-acid therapy also demonstrated T_{max} occurring within an hour (27-40 min), but cumulative 1-hour C^{14} breath excretion was much less (0.06%-0.35%) (Table 2). Folic-acid-deficient or folic-acid-blocked patients in relapse, on the other hand, demonstrate a markedly prolonged T_{max} (140-240 min) and markedly decreased cumulative 1-hour C^{14} breath excretion (0.06%-0.22%) (Table 2).

DISCUSSION

Cumulative pulmonary and renal excretion of C^{14} by two normal subjects during the first month after intravenous administration of L-histidine-2(ring)-C^{14} approximates 45% (Fig. 1). This is somewhat slower than the excretion of C^{14} from glycine-2-C^{14} which approximates 75% during the first month after administration (35). The pattern of excretion for C^{14}-histidine is quite similar to that of C^{14}-glycine. Should continuing measurements establish that this similarity is maintained, then the average tissue radiation following administration of 100 μC of C^{14} as histidine-2(ring)-C^{14} would within a year decrease below the average natural terrestrial and cosmic radiation level. (The maximum permissible total-body burden of C^{14} for occupational exposure is 400 μC as recommended by the National Committee on Radiation Protection, U.S. National Bureau of Standards Handbook 69, 1959.) With the 25-μC dose used in this study, C^{14} tissue radiation corresponds to the natural radiation level at approximately

Table 2. Pulmonary C^{14} excretion in normal subjects and patients with megaloblastic anemia measured during relapse, reticulocyte response and remission

Subject	*	Status	Hb g/100 ml	Reticulocytes %	Bone marrow	T_{max} min	Cumulative 1-hr breath C^{14} %
Control 1	1	normal	14.5	0.1	---	30	1.18
Control 2	1	normal	14.0	0.8	---	50	1.12
M. F.	3	remission on vitamin B_{12}	12.1	1.0	normal	29	1.34
W. C.	2	remission on vitamin B_{12}	15.0	1.0	---	22	0.79
G. M.	3	remission off amethopterin	10.9	0.3	normal	50	1.02
M. F.	1	vitamin B_{12} deficient	9.0	0.9	megaloblastic	40	1.16
J. B.	1	vitamin B_{12} deficient	4.2	0.1	megaloblastic	40	0.71
C. K.	1	vitamin B_{12} deficient	11.7	1.6	megaloblastic	38	0.73
M. F.	2	responding to vitamin B_{12}	10.0	13.8	normoblastic eryth. hyperplasia	28	0.12
W. C.	1	responding to vitamin B_{12}	12.4	7.9	normoblastic eryth. hyperplasia	22	0.05
G. M.	2	responding to absence of amethopterin	9.1	11.6	normoblastic eryth. hyperplasia	30	0.32
J. B.	2	responding to vitamin B_{12}	6.9	10.0	normoblastic eryth. hyperplasia	22	0.06
J. B.	3	responding to vitamin B_{12}	7.3	2.3	normoblastic eryth. hyperplasia	35	0.27
M. G.	1	responding to folic acid	6.5	2.2	normoblastic eryth. hyperplasia	40	0.35
F. M.	2	responding to folic acid	6.1	21.2	normoblastic eryth. hyperplasia	27	0.25
R. M.	1	folic acid-deficient	4.5	0.3	megaloblastic	240	0.06
F. M.	1	folic acid-deficient	6.2	1.0	megaloblastic	140	0.14
G. M.	1	folic acid-block	5.8	1.0	megaloblastic	185	0.22

*Sequential order of the studies in each subject.

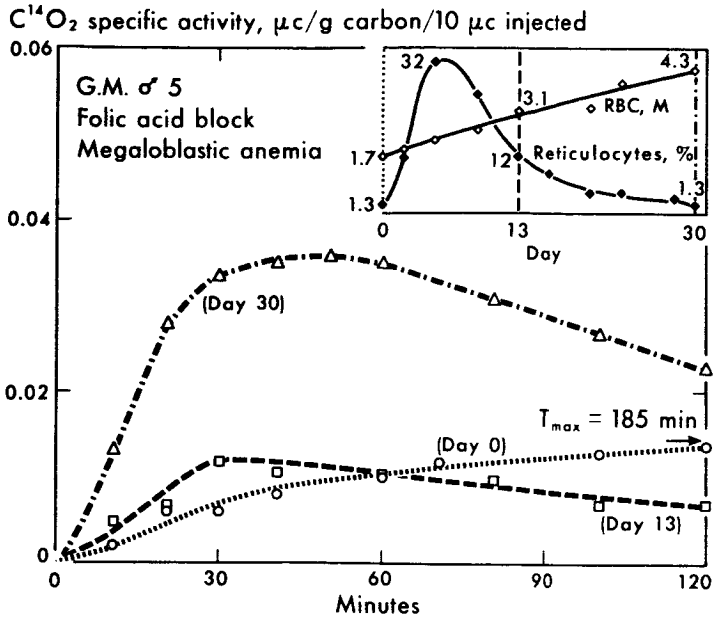

Figure 4. $C^{14}O_2$ specific activity after intravenous administration of C^{14}-histidine to a patient with megaloblastic anemia occurring as a result of amethopterin therapy; studies were performed during relapse (Day 0....), reticulocytosis in response to cessation of amethopterin therapy (Day 13 ----), and remission (Day 30 -.-.-.-.) with respect to the megaloblastic anemia.

MU-27590

4 months. It thus appears that carbon 14, despite its very long physical half-life (5,600 yr), when in the 2(ring) position of histidine, possesses a relatively short biological half-life that permits safe usage for clinical investigation and diagnosis.

The metabolic fate of the 2(ring)-carbon atom of histidine, which was labeled with C^{14} in the studies presented, is shown in Fig. 5. Histidine is metabolized to formiminoglutamic acid (FIGlu) (22), and the labeled carbon atom now resides in the formimino group. This group is then transferred to tetrahydrofolic acid (FH_4), the metabolically active form of folic acid. This transfer coupled with the release of the formimino nitrogen results in the formation of C^{14}-labeled methenyl as N^5,N^{10}-methenyl tetrahydrofolic acid (N^5,N^{10}-methenyl-FH_4) which is one of the metabolically active forms of the monocarbon moiety. This single carbon unit attached to FH_4 may be utilized as 1. a formyl group or its anhydro-derivative, methenyl, 2. a hydroxymethyl group, or its anhydro-derivative, methylene, or 3. a methyl group--depending upon the degree of reduction of this group. It is felt by some researchers that the anhydro-derivatives are probably the active coenzymes (20). The fate of this active monocarbon moiety is multiple. It may be coupled with glycine to form serine which in turn, after loss of the amine group, can be oxidized via the tricarboxylic-acid cycle to CO_2, thus labeling the body CO_2 pool with $C^{14}O_2$. The active monocarbon unit is used in the formation of methionine from homocystine as it appears in methionine as the methyl group. Three methionine molecules in turn transfer their methyl groups to ethanolamine, probably as phosphatidyl ethanolamine (36), a decarboxylated metabolite of serine, to form choline. Betaine, the oxidation product of choline, can return its methyl groups to the active single-carbon-moiety pool,

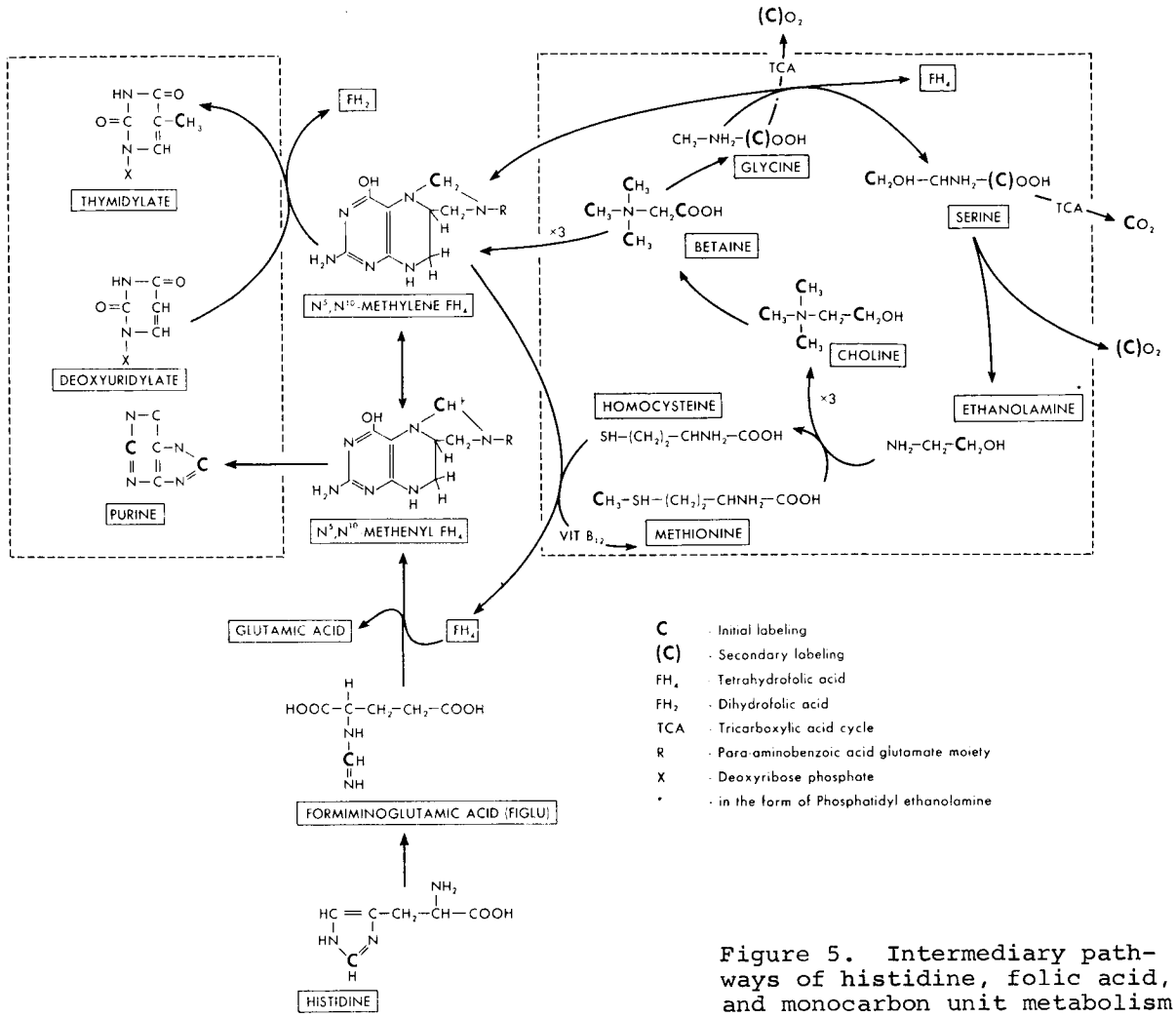

Figure 5. Intermediary pathways of histidine, folic acid, and monocarbon unit metabolism. MUB-1235

with the formation of labeled glycine. This labeled glycine molecule in turn may be oxidized to CO_2, another site of formation of $C^{14}O_2$, or it may combine with still another active single-carbon moiety to form serine, which during its decarboxylation to ethanolamine affords another site of $C^{14}O_2$ formation. As shown in Fig. 5, the active single-carbon moiety is also essential for nucleoprotein synthesis, since it is required for incorporation into the 2 and 8 positions of purine and the methyl group of thymidylate. The essential role of folic acid in metabolism is evident; it is the necessary acceptor for the single-carbon moiety from histidine and other donors (21) and the sole means of transferring this single-carbon moiety in the reactions described above. It is also noted that this labeled 2(ring)-carbon atom of histidine is initially involved in two simultaneous and divergent metabolic pathways: 1. the eventual oxidation to $C^{14}O_2$ and 2. the incorporation into nucleoprotein.

The T_{max} occurring within 1 hour and the cumulative one-hour C^{14} breath excretion of approximately 1% noted in normal subjects, in patients in remission, and in the vitamin-B_{12}-deficient patients in relapse (Table 2) suggest that the quantitative partition of the 2(ring)-carbon atom of histidine between the above described metabolic pathways is similar in each of these groups of subjects. The markedly delayed T_{max} and low cumulative one-hour C^{14} breath excretion noted in the folic-acid-deficient patients in relapse (Table 2) are explained by the marked reduction in amount and rate of transfer of the single-carbon moiety to that portion of the metabolic pathway allowing for production of $C^{14}O_2$. Deficiency of this essential vitamin does not permit release of the formimino carbon atom from FIGlu. The similar results, noted in the patient with the megaloblastic anemia (Table 2, Fig. 4) while receiving amethopterin, lend support to this explanation as it appears that the antifolic action of this drug is to prevent the conversion of FH_2 to FH_4 (37). A low specific-activity curve with T_{max} occurring normally within 1 hour and decreased cumulative 1-hour C^{14} breath excretion were noted consistently during response to vitamin therapy (Table 2; Figs. 3 and 4). The occurrence of this pattern during increased normal nucleoprotein synthesis may be explained by the increased incorporation of the labeled metabolically active single-carbon moiety into purine and pyrimidine, leaving a relatively small amount of the active single-carbon moiety available for oxidation to $C^{14}O_2$.

The specific-activity curves obtained in vitamin-B_{12}-deficient patients with megaloblastic anemia demonstrate a normal T_{max} and normal cumulative 1-hour C^{14} breath excretion. The normal values for T_{max} are to be expected since the active monocarbon units are made available to oxidative pathways at a normal rate of transfer from FIGlu in the presence of adequate folic acid. The unanticipated normal cumulative 1-hour C^{14} breath excretion provokes several interesting speculations. While there is accumulating convincing evidence that vitamin B_{12} is required for methionine-methyl neogenesis (Fig. 5) and deoxyribose formation (20), the latter essential in DNA synthesis, no increase of breath C^{14} excretion was noted in these vitamin-B_{12}-deficient patients in relapse. Conversely, despite the greatly increased number of giant myeloid cells, megaloblasts, and megaloblastic nucleated red cells with normal or increased DNA content in the marrow (38), many of which were undergoing rapid intramedullary destruction (39,40,41,42,43,44), and similar cytologic changes in other body cells (45,46, 47,48), breath C^{14} excretion was not decreased. Thus, despite greatly increased DNA synthesis, either the total number of monocarbon units required for purine and pyrimidine synthesis in patients with vitamin-B_{12}-deficient megaloblastic anemia is not increased; or, as seems more probable, large numbers of monocarbon units are supplied <u>in situ</u> as a result of abnormal cell catabolism and recycling so that the <u>additional</u> number of monocarbon units required from the "central" metabolic pool for purine and pyrimidine synthesis is approximately equal to the total number normally required.

Herbert and Zalusky have suggested that in vitamin-B_{12}-deficient subjects, "piled up" L. casei-active folate activity ("probably N^5-methyl-tetrahydrofolic acid") "would tend to reduce the amount of folic acid available for other 1-carbon unit transfers" (49). The tracer studies here presented, however, demonstrate that in vitamin-B_{12}-deficient patients with megaloblastic anemia, folic acid is not decreased sufficiently to reduce the rate of transfer of the 2(ring)-carbon atom of histidine to the active monocarbon pool nor to reduce appreciably monocarbon-unit transfer from FH_4 to oxidative pathways.

Finding of normal $C^{14}O_2$ production in vitamin-B_{12}-deficient patients and markedly reduced $C^{14}O_2$ production in folic-acid-deficient patients contrasts with the demonstration of markedly reduced $C^{14}O_2$ production both in vitamin-B_{12}- and folic-acid-deficient rats by Brown, Silva, Gardiner, and Silverman (26). These weanling rats were judged vitamin deficient when urinary FIGlu became increased after 4 weeks of feeding on a synthetic diet in which the appropriate vitamin was omitted. The diet contained 9% casein, thereby limiting the sulfur-amino acids, and was fortified with 0.2 per cent L-histidine. Species difference, special diet, absence of megaloblastic anemia, and lack of evidence of vitamin deficiency, except increased FIGlu excretion, make comparison difficult.

Measurement of urinary FIGlu is the only previously used differential diagnostic test that provides some insight into the nature of the metabolic derangement present in the individual patient. Although the determination of breath $C^{14}O_2$ and the measurement of urinary FIGlu after histidine loading permit examination of related aspects of the same metabolic process, two important distinctions can be made between these investigative approaches. The current study uses only a tracer dose of 0.5 milligrams histidine as compared with the 2,000-20,000 milligrams of histidine used in the urinary FIGlu test. This large unphysiologic dose of histidine may be readily limited in its metabolism by retrograde accumulation of intermediates, including FIGlu, as the result of an enzyme deficiency such as vitamin B_{12} that would limit the rate of a step subsequent to the transfer to the formimino group from FIGlu to tetrahydrofolic acid. The considerable overlap of urinary FIGlu excretion, observed in patients with folic-acid deficiency and patients with vitamin-B_{12} deficiency, may be a consequence of the large loading dose of histidine. Perhaps little or no overlap of urinary C^{14}-FIGlu values will be observed between these two groups following the administration of tracer amounts of C^{14}-histidine.

Continuous measurement of breath $C^{14}O_2$ discloses two significant parameters, T_{max} and cumulative C^{14} excretion, as compared with the single parameter of the amount of FIGlu excreted in the urine. The time interval between injection and maximal $C^{14}O_2$-specific activity, T_{max}, appears to be a sensitive index of the oxidation rate of the 2(ring)-carbon atom of L-histidine to $C^{14}O_2$.

This oxidation involves the transference of the formimino moiety from FIGlu to tetrahydrofolic acid. Hence folic-acid deficiency, but not vitamin-B_{12} deficiency, causes marked prolongation of T_{max}. If T_{max} is normal, then cumulative C^{14} breath excretion seems to be a significant index of the partition of the active monocarbon moieties between pathways for oxidation and synthetic pathways resulting in the formation of purines and pyrimidines. If these preliminary results were confirmed in a large series of patients, measurements of these parameters would provide specific criteria for rapid differentiation between patients with vitamin-B_{12}- or folic-acid-deficient megaloblastic anemia. In addition, and more basically, this approach provides a quantitative dynamic representation of metabolic function or malfunction, which will be more complete when integrated with measurements derived from current analyses of blood and urine samples.

SUMMARY

Intermediary metabolism of the monocarbon pool and histidine in normal subjects and patients with megaloblastic anemia were studied by continuous measurement of pulmonary excretion of $C^{14}O_2$ and urinary excretion of C^{14} after injection of L-histidine-2(ring)-C^{14}. Cumulative pulmonary and renal excretion of C^{14} for one month by two normal subjects approximates 45% of the amount injected. Within 4 months after injection of the dose used in this study, the resultant average tissue radiation decreases below the average natural terrestrial and cosmic radiation level.

Simultaneous determination of two parameters, 1. cumulative 1-hour pulmonary C^{14} excretion and 2. the time of occurrence of maximum $C^{14}O_2$ specific activity (T_{max}), may permit rapid and unequivocal differentiation between folic-acid deficiency and vitamin-B_{12} deficiency in the pathogenesis of megaloblastic anemia. Folic-acid deficiency results in marked diminution of pulmonary C^{14} excretion (approximately 0.1% of injection C^{14} in 1 hr) and marked prolongation of $C^{14}O_2$ specific activity T_{max} (approximately 3 hr), while both parameters are normal (approximately 1% and less than 1 hr, respectively) in patients with vitamin-B_{12} deficiency and megaloblastic anemia.

Measurements during periods of reticulocyte response to either folic acid or vitamin B_{12} demonstrate normal $C^{14}O_2$ specific activity T_{max} but decreased pulmonary C^{14} excretion. These observations suggest that prolongation of $C^{14}O_2$ specific activity T_{max} is a sensitive index of folic-acid deficiency or block and that if T_{max} is normal, pulmonary C^{14} excretion is a sensitive index of the relative partition of the active monocarbon pool between pathways for oxidation and pathways for nucleic-acid synthesis.

This type of breath analysis seems to provide a quantitative dynamic representation of metabolic function which may be particularly useful in

differentiating between the alterations of intermediary metabolism that occur in patients with folic-acid-deficient megaloblastic anemia and in patients with vitamin-B_{12}-deficient megaloblastic anemia

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Autoradiographic Evidence for a Pluripotential Stem Cell in the Bone Marrow

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Most hematologists agree that the various types of bone-marrow cells arise from primitive undifferentiated stem cells. However, considerable controversy exists regarding the developmental potentialities of individual stem cells; some investigators maintain that the stem cell is pluripotential, whereas others believe that each type of blood cell is derived from a single stem cell whose line of development has been previously determined. Still others have proposed intermediate theories. These theories cannot be substantiated by morphological methods alone, but some evidence for the pluripotential theory has been obtained in the present experiments utilizing autoradiography.

Forty female Swiss mice, weighing approximately 22 g each, were hypertransfused by the intravenous injection of an 80% suspension of red blood cells. The red blood cells, obtained by cardiac puncture from donor Swiss mice, were washed three times in saline and filtered through a nylon mesh before injection. The recipient animals were divided into four groups and injected on day 1 and day 2 and then again on day 11 with the red blood cells. Two groups of animals were irradiated on day 7 with 150 r (200 kV, 15 mA, Cu 0.25, Al 1.0, dose rate of 35 r/min). Starting on day 11 through day 13 all groups of animals were injected intravenously with 0.5 μC/g of H^3-thymidine (1.9 C/mM), every 12 hr for a total of 6 injections. Six hours after the last H^3-thymidine injection, one irradiated and one nonirradiated group of mice received an intravenous injection of 6 cobalt units of sheep erythropoietin (Armour Lot No. K103194A obtained from the Hematology Study Section, National Institutes of Health).

For the first 3 days after the injection of erythropoietin mice were sacrificed at approximately 12-hr intervals. The hematocrits of all mice were >60% at the time of this sampling. Two irradiated and nonirradiated mice were sacrificed just prior to the injection of erythropoietin, and two mice from each of the four groups were sacrificed 12 days after the injection of erythropoietin. Smears were made of the femoral bone marrow of all the mice and sections made of the spleen after Bouin fixation. Autoradiographs of these smears and sections were made using both the dipping and stripping-film techniques. Some of the smears were stained for hemoglobin using Ralph's stain (1) before making the autoradiographs. These were then exposed for 3 weeks, developed in D170,

fixed, and stained with Giemsa at pH 6.8. In a separate experiment, groups of mice were injected with erythropoietin or severely bled, received multiple injections with H^3-thymidine as before, and were then hypertransfused. The hypertransfused state was maintained at a hematocrit >70% for 4 weeks. The mice were then injected with erythropoietin, and 2 days later smears were made of the bone marrow. Autoradiographs of these smears were then made.

RESULTS

During the first 3 days after the last injection of H^3-thymidine, almost all of the cells in the bone marrow of the different groups of mice were labeled, i.e., granulocytic, megakaryocytic, and erythrocytic cell series. Significant numbers of nucleated erythroid cells did not appear in the bone marrow of hypertransfused mice until nearly 24 hr after the erythropoietin injection, or 30 hr after the last injection of H^3-thymidine. During the subsequent maturation of cells in the initiated wave of erythropoiesis, it was rare to find an erythroid cell that was not labeled, in fact we found that even the most mature nucleated red cells were labeled on the 3rd day after erythropoietin injection. During maturation a decreased number of silver grains was observed over the various types of cells. By the 12th day after the last H^3-thymidine injection, labeled myeloid cells were rarely found. Interestingly, the labeling patterns in the bone-marrow cells of the mildly irradiated and the nonirradiated group of mice were not different qualitatively. In the mice which had been labeled by multiple injections of H^3-thymidine before hypertransfusion, and the elevated hematocrit maintained for 4 weeks, we found no labeled cells before or after the injection of erythropoietin.

Filmanowicz and Gurney (2) have shown that the spleen of the hypertransfused mouse is essentially devoid of identifiable erythroid cells. They further showed that stimulation of such animals with exogenous erythropoietin produces an orderly wave of erythropoiesis. A similar wave of erythropoiesis is found in the bone marrow (3), and this was observed in the present experiment. The wave is seen as peak percentages of immature proerythroblasts 1 day after erythropoietin injection followed by mature erythroblasts at 2 days and eventually as reticulocytes at 3 days.

The fact that a population of labeled erythroid cells was produced in the bone marrow of hypertransfused mice 30 hr after the injection of H^3-thymidine raises several questions. What was the immediate source of label for these erythroid cells? The presence of free H^3-thymidine 6 hr after H^3-thymidine injection can be excluded, because the availability time of this label is very short. It seems unlikely that reutilization of the label could account for the total labeling of the rather large population of labeled erythroid cells found after erythropoietin stimulation. Thus, it would appear that labeling of some stem cell which cannot be morphologically distinguished as an erythroid cell

occurs in hypertransfused mice, even though active erythropoiesis is not evident. If only a few labeled stem cells had been stimulated by erythropoietin to become erythroid cells, and this stimulation caused proliferation of the stem cell, the label should have been rapidly diluted out during erythroid maturation. Since such a dilution did not occur, and nearly all nucleated erythroid cells were labeled during the entire wave of erythropoiesis, it appears more likely that erythropoietin stimulated the differentiation of a large number of labeled stem cells. Immunologic studies also suggest that erythropoietin controls erythropoiesis by regulating the differentiation of stem cells (3).

The availability of such a large population of labeled stem cells at the time of erythropoietin injection indicates that most of these stem cells had passed through a DNA synthetic phase during the time when H^3-thymidine was present. This seemed rather surprising inasmuch as we had assumed that the stem-cell population was turning over rather slowly in hypertransfused animals and in the experiments had mildly irradiated some groups of mice in order to cause an increased proliferation of the stem-cell compartment, during the H^3-thymidine injections. Gurney (4) finds that even a single injection of H^3-thymidine into hypertransfused mice labels some stem cells which upon subsequent stimulation with erythropoietin differentiate into labeled erythroid cells. Gurney's finding and ours indicate that considerable proliferation of the stem-cell pool occurs even in the absence of active erythropoiesis.

In spite of the fact that considerable proliferation of the stem-cell compartment occurs in hypertransfused mice, there is no apparent accumulation of reticulum cells. We have seen, however, a relative increase in the numbers of small lymphocyte-like cells. Labeled cells of any type are not seen in the bone marrow in hypertransfused mice 2 weeks after multiple injections of H^3-thymidine. Thus, it appears that labeled stem cells which have not been stimulated to differentiate do not simply remain dormant in the marrow. This is further substantiated by the finding that mice whose erythropoiesis had been stopped for a month following H^3-thymidine injections did not produce labeled erythroid cells after erythropoietin stimulation. The most reasonable conclusion is that the labeled stem cells of the hypertransfused mouse are stimulated to differentiate into other cell lines such as granulocytic or megakaryocytic cells, and that the maturation of these cell lines dilutes the label which eventually cannot be detected. These results are consistent with the model of erythropoiesis proposed by Lajtha, Oliver and Gurney (5) only if the stem cell is pluripotential. We cannot exclude, however, the possibility of independent stem-cell populations which actively proliferate and die if not stimulated to differentiate.

At the present time, the morphological identity of the stem cell cannot be established with certainty. However, of all the cell populations in the erythroid marrow, that of the small round cells seems to be the only one ade-

quately labeled and numerically large enough to subsequently differentiate into a large population of labeled erythroid cells upon erythropoietin stimulation. Morphological separation of these round cells from small lymphocytes is very difficult; however, Schooley, Bryant and Kelly (6) previously have shown that the pattern of labeling of these cells is different from that of the small lymphocyte in the lymph nodes and peripheral blood. More recent work has confirmed this earlier observation.

CONCLUSION

We suggest from these experiments that there is considerable proliferation in the stem-cell compartment of hypertransfused mice, and that upon appropriate stimulation these stem cells can differentiate into either granulocytic, megakaryocytic, or erythrocytic cells. This stem cell is most likely the small round cell of the bone marrow.

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